

FLAVONOID PROTECTION OF CARDIAC CELLS AGAINST ISCHEMIA-REPERFUSION INJURY

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ABSTRACT

Myocardial ischemia-reperfusion injury occurs following the majority of cardiac events including myocardial stenosis and heart surgeries. As reactive oxygen species are one of the major contributors to ischemia-reperfusion injury, strategies to prevent their effects may be directed towards enhancing the antioxidant capacity of cells. Polyphenols, and in a more specific category, flavonoids are strong antioxidants, while possessing other biological activities such as anti-apoptotic, anti-inflammatory, and vasodilatory effects.

I hypothesized that flavonoids are able to reduce ischemia-reperfusion-induced cell death through multiple mechanisms including reduction of oxidative stress and induction of cellular antioxidant enzymes. The hypothesis was tested in *in vitro* and *in vivo* phases.

In the first phase of the studies, rat embryonic ventricular H9c2 cells were treated with various concentrations of polyphenols with or without ascorbate for 1-3 days before induction of ischemia and reperfusion. Ischemia was induced by exposure of the cells to a non-glucose containing solution bubbled with nitrogen, and reperfusion by returning the regular medium containing the corresponding polyphenols and/or ascorbate. Cell viability measurements using the MTT assay or counting acridine orange-stained cells showed that the best protection against cell death was given by catechin (44-58 %), epigallocatechin gallate (48%), proanthocyanidins (44%), and ascorbic acid (57-92%). A low concentration (10 μ M) of catechin was more effective with a long-term (2 days) incubation time (64%), while a higher concentration (50 μ M) could exert benefit even after 1 h pre-treatment (98%). Quercetin, resveratrol, cyanidin, and delphinidin displayed almost no protection.

In the second part of the *in vitro* study, H9c2 cells were treated with 350 to 450 μ M *tert*-butyl hydroperoxide for 24 h after pre-incubation with various concentrations of polyphenols with or without ascorbate for either short (1 h) or prolonged (3 days) periods. Unlike in the ischemia-reperfusion experiments, 3 days pre-treatment with polyphenols did not protect and often caused cytotoxicity. In short-term (1 h) pre-treatments, the best protection was obtained with 50 μ M quercetin (95%), 50 μ M epigallocatechin gallate (66%), and 100 μ M catechin (28%). Pre-treatment with ascorbic acid (100 μ M) with or without polyphenols did not improve cell survival except in one case where it enhanced cytoprotection by epigallocatechin gallate.

The second phase of the studies was performed with isolated rat hearts. Rats were fed diets containing broccoli sprouts (2%), saskatoon berries (5%), or green tea extract (0.25%) for 10 days before induction of global ischemia for 20 min and reperfusion for 2 h. Broccoli sprouts decreased cell death in ischemic-reperfused hearts as assessed by caspase-3 activity (86%) and DNA fragmentation (78 %), attenuated oxidative damage as detected by lower thiobarbituric acid reactive substances (TBARS) (116%) and preserved aconitase activity (82%). Green tea extract prevented apoptosis in hearts as detected by caspase-3 activity (85%), but did not inhibit DNA fragmentation. Berries showed lower TBARS (73%). None of the feedings significantly prevented necrosis as evaluated by the release of lactate dehydrogenase into the coronary effluents, improved coronary flow, or increased heart glutathione.

Green tea extract was the only intervention capable of preserving the activity of glutamate cysteine ligase (78%) and quinone reductase (147%) in hearts. The sprouts group was the only group which induced these same enzymes in liver (40 and 44 %, respectively), as it was the only intervention which elevated total liver glutathione (12%). None of the interventions changed heme oxygenase-1 protein levels. Assessment of total polyphenol content revealed that broccoli sprouts had the lowest and green tea extract had the highest amount of polyphenols among the three plant materials, suggesting that the protection exhibited by broccoli sprouts was unlikely to be due to the polyphenols.

In conclusion, flavonoids and flavonoid-rich foods can strengthen the cellular ability to fight against oxidative stress. A part of this effect could be due to their direct antioxidant activity, while in prolonged applications they may also activate cellular pathways to promote endogenous antioxidant defences of cells. Application of low doses of flavonoids and consumption of flavonoid-rich plants in long-term ensures their effectiveness while avoiding possible toxicity. However, plants such as broccoli sprouts may have other chemical ingredients bearing biological properties which may help cells to survive states of oxidative stress.

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ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane potential
$\cdot\text{OH}$	Hydroxyl radical
ADP	Adenosine diphosphate
AP-1	Activator protein-1
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
DCIP	2,6-Dichloroindophenol
DHA	Dehydroascorbic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCL	Glutamate cysteine ligase
GSH	Glutathione
GSSG	Glutathione disulfide
H ₂ O ₂	Hydrogen peroxide
HO-1	Heme oxygenase-1
ICAD	Caspase-activated DNase inhibitor
ICAM	Intercellular adhesion molecule
IL	Interleukin

IP3	Inositol triphosphate
iNOS	Inducible nitric oxide synthase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LTB₄	Leukotriene B ₄
MDA	Malondialdehyde
MnSOD	Manganese superoxide dismutase
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
Na asc	Sodium ascorbate
NAD	Nicotinamide adenine dinucleotide
NADP⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDA	2,3-Naphthalenedicarboxyaldehyde
NF-κB	Nuclear factor-kappaB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NQO	NAD(P)H:quinone oxidoreductase
Nrf2	Nuclear factor-E2-related factor-2
O₂^{•-}	Superoxide radical
ONOO⁻	Peroxynitrite
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PTP	Permeability transition pore
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TBARS	Thiobarbituric acid reactive substances
<i>t</i>-buOOH	<i>tert</i> -butylhydroperoxide
TNF-α	Tumor necrosis factor-α
TXA₂	Thromboxane A ₂

UDP	Uracil diphosphate
VCAM	Vascular cell adhesion molecule
VDAC	Voltage-dependent anion channels

CHAPTER 1

INTRODUCTION

1.1. Rationale

According to the World Health Report of the World Health Organization (WHO) 2003, cardiovascular diseases including coronary (ischemic) heart disease, stroke, and hypertension are the leading cause of death worldwide, making up to 29.2% of the total deaths in the world. Ischemic heart disease makes a contribution of 12.6%, still the highest culprit of death, among all cause mortalities. Heart disease was also the major cause of death in the U.S. in 2003 (U.S. National Vital Statistics Reports). In Canada, cardiovascular diseases including coronary heart disease, cerebrovascular disease, and raised blood pressure ranked first among the leading causes of death in 1997, making 36.8% of deaths in the country. However, heart diseases were the second major cause of death after cancer.

Myocardial ischemia-reperfusion injury occurs following a number of medical situations including stenosis, angioplasty, and cardiac surgeries which in severe cases can result in serious heart morbidities. Heart failure is one of the aftermaths of ischemia-reperfusion injury that in many cases a few years later terminates in death. Heart failure generally originates from death of myocardial cells due to pathological alterations resulting from ischemia and reperfusion. After death, leftovers of the dead cells are removed by phagocytes and neighbouring cells. Thereafter, as a part of the repairing process, myofibroblasts deposit collagen in the vacated area (Virag & Murry, 2003; Petrovic, 2004). Excessive synthesis of collagen can lead to thickening of the ventricular wall and ventricular remodeling (Heeneman et al., 2003). This reparative process, although advantageous in the short term as it prevents ventricular wall thinning and dilation, ultimately results in tissue stiffness, ventricular dysfunction and heart failure (Vinten-Johansen, 2007). In fact, the extent of ventricular remodeling reflects the size of infarction, which is one of the important determinants of the heart failure. Thus, limiting myocardial cell death is one of the strategies to attenuate post-ischemic injury and heart failure and to reduce the eventual mortality.

Ischemia preconditioning has been introduced as one the most powerful cardioprotective interventions against ischemia-reperfusion injury (Bolli, 2007). Preconditioning means enhancing the strength of the heart by a mild stress in order to better withstand a next-coming injurious insult. The mechanisms of preconditioning are not thoroughly understood, but it is postulated that alterations in gene expression and/or protein activity are involved. Preconditioning may be achieved by pharmacological and non-pharmacological strategies. One of the pharmacological schemes for heart preconditioning is administration of flavonoids, a group of plant compounds whose cardioprotective properties have attracted attention. Numerous studies have succeeded to precondition the heart or cultured cardiomyocytes with various types of flavonoids (are reviewed below), and the outcomes have been promising.

Flavonoids, which are a part of the more extended family of polyphenols, are well known for their antioxidant activities. They exert antioxidant effects by scavenging reactive oxygen species (ROS) (Jovanovic & Simic, 2000), chelating transition metals (Mandel et al., 2006), and inhibiting enzymatic generation of ROS (Cos et al., 1998; Kim et al., 2004). Also, a part of the antioxidant activity of flavonoids is exerted through enhancement of phase 2 enzymes (Moon et al., 2006). Cardioprotective effects of flavonoids can also be attributed to their vasorelaxant (Zenebe et al., 2003), anti-inflammatory (Kim et al., 2004), and anti-platelet aggregation (Cos et al., 2004) characteristics.

Our main goal was to evaluate the potential of flavonoids to precondition cardiac cells against ischemia-reperfusion injury. Explanations below will describe what our study is going to add to the literature. The study contained *in vitro* and *in vivo/ex vivo* experiments.

1.1.1. *In vitro* studies

Anti-apoptotic capacity of some flavonoids against a variety of insults especially oxidative stress has been indicated by many investigators (Watjen et al., 2005; Ruiz et al., 2006; Mandel et al., 2005). However, the literature lacks information on whether some flavonoids are more protective than others. The availability of such knowledge to investigators helps to introduce more effective compounds for application in animal studies, although discovering the potential of a specific compound in inhibiting cell demise *in vitro* does not necessarily mean that it can show cardioprotection in animals and particularly humans. The initial intention was to do a comparison between anti-apoptotic effects of a

relatively extensive variety of flavonoids in ischemic-reperfused cardiomyocytes. However, later when the model of ischemia-reperfusion on the cardiomyocytes produced inconsistent results, the experiments were continued with hydroperoxides and the anti-apoptotic effect of the same flavonoids was tested in cardiomyocytes subjected to hydroperoxides.

1.1.2. *Ex vivo* studies

During the past 3 decades, enormous attempts have been made in experimental settings especially animal studies examining hundreds of pharmacological and non-pharmacological interventions to prevent or at least attenuate myocardial ischemia-reperfusion injury (Bolli et al., 2004). However, none of these interventions has been accepted as a standard treatment in clinical practice. One of the substantial reasons for this failure in translation of laboratory-effective interventions into standard medical procedures is the time of application. In clinical studies the interventions are mainly applied late in the ischemia or early in the reperfusion, whereas in experimental studies the interventions are administered prior to the ischemia. This disconnect between research and practical contexts arises from the unpredictable essence of some cases of myocardial ischemia-reperfusion injury. The unpredictable nature poses the necessity of interventions that can be recommended to high-risk but otherwise normal populations without exposing them unnecessarily to adverse consequences of the intervention.

In investigating the cardioprotective potential of flavonoids, many studies have used flavonoids in the form of pure compounds administered via feeding, injection, or perfusing hearts with doses that generally cannot be achieved by a regular diet. Administration of high doses of flavonoids poses the possibility of toxicity. This raises the question of whether the cardioprotective effects of flavonoids can be exerted by low naturally available concentrations of flavonoids in foods. If flavonoid-rich foods can provide cardioprotection against ischemia-reperfusion injury, at least two benefits will be attained over their administration in pure form. On one hand, the likelihood of the flavonoid toxicity will decrease. Since the concentration of flavonoids in foods and their bioavailability at the absorption site is low, they are less likely to be deleterious if they are given in their naturally occurring context, whereas application of the high doses of pure compounds may put the body at the risk of toxicity. On the other hand, if flavonoid-rich foods are proved effective,

it is quite feasible to benefit from their cardioprotective effects in generally at risk populations such as elderly individuals or diabetics without requiring them to take possible medications.

1.2. Hypothesis

Flavonoids *in vitro* and in the diet are able to protect cardiac cells and reduce cell death caused by ischemia-reperfusion injury through multiple mechanisms including reduction of oxidative stress, improved cellular antioxidant capacity, induction of antioxidant enzymes, and enhancement of cellular ATP levels.

1.3. Objectives

Objective 1. To evaluate the ability of polyphenols to protect cultured cardiomyocytes from ischemia-reperfusion injury *in vitro*

Specific objectives were:

- 1.1.** To establish the *in vitro* model of ischemia-reperfusion that could induce cell death in rat embryonic ventricular H9c2 cells
- 1.2.** To determine the extent to which various selected polyphenols can protect rat embryonic ventricular H9c2 cells and reduce cell death against simulated ischemia-reperfusion *in vitro*
- 1.3.** To examine whether ascorbate improves polyphenol protection of H9c2 cells against *in vitro* ischemia-reperfusion injury

Objective 2. To evaluate the ability of polyphenols and protect H9c2 cells against oxidative stress induced by hydroperoxides

Specific objectives were:

- 2.1.** To assess the ability of various selected polyphenols to increase cell viability in H9c2 cells subjected to *tert*-butyl hydroperoxide
- 2.2.** To determine whether ascorbate can improve protection by polyphenols of H9c2 cells against *tert*-butyl hydroperoxide

Objective 3. To investigate whether feeding rats with flavonoid-rich foods protects their hearts against ischemia-reperfusion injury *ex vivo*

Specific objectives were:

- 3.1.** To determine whether 10 days feeding with saskatoon berries, green tea extract, or broccoli sprouts decreases cardiac cell death induced by ischemia-reperfusion in isolated rat hearts
- 3.2.** To examine whether the above-mentioned feeding strategies mitigate oxidative stress-induced biochemical alterations in hearts
- 3.3.** To determine whether the above mentioned feedings improve glutathione content in heart and liver tissues
- 3.4.** To evaluate whether the feedings enhance the protein expression and/or activity of phase 2 enzymes in heart and/or liver tissues

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Ischemia, or inadequate blood flow, in heart occurs following vascular occlusion upon unwanted cardiac events such as heart attack or deliberate medical purposes such as angioplasty and coronary bypass surgery. During ischemia, cardiac cells, which are in constant need of oxygen and glucose, may seriously be injured and face death. Restoration of blood flow, so-called reperfusion, although the exclusive strategy to salvage the myocardial cells from ischemic death, can adversely impact their recovery. Accordingly, the resulting damage is called ischemia-reperfusion injury.

Reperfusion after a period of ischemia may be more damaging than the same period of ischemia alone. For instance, in dogs 40 min coronary occlusion followed by 20 min reperfusion caused more damage than 60 min occlusion alone, evidenced by glycogen depletion, contraction bands, alteration in intracellular ionic concentrations, and cellular and mitochondrial swelling (Shen & Jennings, 1972). None of these changes was evident after 60 min ischemia without reperfusion. Similarly, 60 min global heart ischemia in rabbits did not change the appearance of cells in the right ventricular wall, but 5 min reperfusion following the same course of ischemia was accompanied with cell swelling and manifestations of plasma membrane rupture (Walsh & Tormey, 1988).

2.2. Myocardial cell death in ischemia-reperfusion injury

The appearance of dead cells following ischemia-reperfusion in human hearts is well documented. Olivetti et al. (1996) studied cardiac tissues from ischemic patients who died within 10 days from the onset of myocardial infarction symptoms. Apoptosis was detected as early as 12 h after ischemia and apoptotic cells were still detectable 10 days afterwards. Quantitatively, 12% of cardiac cells in the adjacent region and 1% of cells in the area remote from the necrotic tissue were apoptotic. It is worth noting that the initial insult may not

necessarily cause an immediate cell death, but it could serve to trigger intracellular production of reactive oxygen species and cellular signaling leading to eventual cell death (Han et al., 2004).

There is debate on which form of cell death, apoptosis or necrosis, predominates after episodes of ischemia-reperfusion. While some scientists have introduced necrosis as the major form of myocardial cell death (Zhao & Vinten-Johansen, 2002), there is evidence in favour of apoptosis as well (Kajstura et al., 1996). It has been suggested that apoptosis primarily occurs during reperfusion rather than ischemia (Zhao & Vinten-Johansen, 2002). Zhao and coworkers (2000) reported the existence of apoptotic cells after 1 h ischemia and 6 h reperfusion, but not after 7 h ischemia alone. Similarly, Gottlieb et al. (1994) reported lack of apoptotic cells in rabbit myocardium after 4.5 h ischemia, while DNA nucleosomal fragmentations were evident after 30 min ischemia and 4 h reperfusion. However, some investigators (Kajstura et al., 1996) have provided evidence supporting the idea that apoptosis can occur during both ischemia and reperfusion. Other investigators document that both ischemia and reperfusion can cause apoptosis; however, reperfusion accelerates apoptosis in non-salvageable cells (Fliss and Gattinger, 1996; Veinot et al., 1997). According to Fliss and Gattinger (1996), reperfusion on one hand inhibits expansion of the apoptotic zone in ischemic myocardium, but at the same time it expedites the progress of apoptosis and therefore removes non-salvageable cells from the tissue. In agreement, Freude et al. (2000) while supporting for the absence of apoptotic cells in ischemic myocardium, provided evidence indicating that the apoptotic form of cell death is initiated during ischemia but reperfusion is needed probably to provide energy for completion of the process.

2.2.1. Forms of cell death

It is more than 150 years that cell death has been recognized as a normal event in living organisms. However, it was not until 1972 that the term “apoptosis” was designated to programmed or suicidal form of cell death by Kerr and colleagues (Kerr et al., 1972). Apoptosis is an energy-dependent process composed of a genetically controlled sequence of events. Apoptosis plays an important role in physiologically normal situations such as eliminating certain cells during development of embryos or cell turnover in adult tissues. Morphological characteristics of apoptosis include cell shrinkage, plasma membrane

budding, chromatin condensation which creates a horseshoe appearance for the nucleus, nuclear fragmentation, and cellular breakage into membrane-bound fragments, also known as apoptotic bodies (Fink & Cookson, 2005; Majno & Joris, 1995; Haunstetter & Izumo, 1998). The apoptotic bodies can then be captured by neighbouring cells where their components are recycled. Splitting cells up into membrane-bound bodies prevents releasing cellular contents into the surrounding environment which could subsequently arouse inflammatory reactions. Biochemical features of apoptosis are activation of cysteine-dependent aspartate-specific proteases (known as caspases), cleavage of DNA into fragments with lengths of multiples of 180- to 200- base pairs which appear on electrophoresis gels as ladder, appearance of phosphatidylserine on the outer side of the plasma membrane (Fink & Cookson, 2005; Majno & Joris, 1995; Haunstetter & Izumo, 1998), and maintenance of ATP levels (Nicotera et al., 1998). The process of apoptosis requires ATP in several steps. Chromatin condensation and nuclear fragmentation are apoptotic steps requiring ATP (Bradbury et al., 2000).

In contrast to apoptosis, necrosis, also called oncosis or accidental form of cell death, is manifested by increased membrane permeability, cellular and organelle swelling, and coagulation of cytoplasmic proteins (Majno & Joris, 1995). As a result of cell swelling, cellular contents and pro-inflammatory molecules are released, provoking inflammatory responses associated with migration and infiltration of macrophages into the necrotic region. However, similar to apoptosis, the cellular debris released from necrotic cells can be engulfed and reutilized by neighbouring cells (Fink & Cookson, 2005). DNA in necrosis is fragmented in random fashion, generating smears rather than ladders in electrophoresis gels (Fliss & Gattinger, 1996). In necrosis, disruption of mitochondrial membrane integrity makes mitochondria incapable of producing ATP which leads to rapid cellular ATP depletion (Yaglom et al., 2003; Halestrap et al., 2000). In fact, cellular ATP levels dictate whether cells die by apoptosis or necrosis (Nicotera et al., 1998). Since apoptotic cells have comparably higher contents of ATP and lower ADP to ATP ratio than necrotic cells, cellular ADP to ATP ratio has been suggested as an indicator for differential recognition of the mode of cell death between apoptosis and necrosis (Bradbury et al., 2000).

Classical apoptosis and necrosis are two foremost forms of cell death. Other types of cell death have also been characterized, each possessing an exclusive pathway and displaying discrete morphological and biochemical manifestations (Fink & Cookson, 2005). Although the mechanisms of various forms differ, they may overlap under pathological

conditions. For instance, it has been shown that internucleosomal DNA fragmentation, which is one of the hallmarks of apoptosis, can also occur during early stages of necrosis, although by a mechanism distinct from that in apoptosis, with random DNA cleavage occurring later in the necrotic process (Dong et al., 1997).

Furthermore, at a single time point in response to a particular noxious stimulus, several modes of cell death may coexist in a system and it is the culmination of the cross-talk between these which eventually determines the primary form of death (Fink & Cookson, 2005). Inhibiting one pathway of cell death may not prevent cell death, but instead activate an alternative mode of death, switching the form of death from one to another (Lockshin & Zakeri, 2004). Factors such as type and intensity of the insult and growth or differentiation state as well as physiological conditions of cells are important in determining the predominant form of death (Fink & Cookson, 2005). For example, in response to an apoptotic stimulus cells may die from necrosis if their energy levels are rapidly depleted (Leist et al., 1997). Likewise, one particular stimulus which induces apoptosis at low concentrations may cause necrosis at higher concentrations (Kruman et al., 1997). It is likely, though, that during ischemia-reperfusion some cells are subjected to severe stress and undergo necrosis, whereas others which are located in the surrounding region proceed to apoptosis (Olivetti et al., 1996). Evidence shows that the magnitude of biochemical alterations is more substantial in necrosis than apoptosis (Kruman & Mattson, 1999).

2.3. Culprits of myocardial ischemia-reperfusion injury

Although the mechanisms responsible for heart ischemia-reperfusion injury have not yet been elucidated in detail, increasing evidence points at two major culprits of biochemical and pathophysiological changes during this clinical event: reactive oxygen species and calcium. These two factors are very likely to collaborate in causing the injury. However, what needs to be clarified is to what extent each contributes to the injury, and whether they occur simultaneously or if one occurs ahead and acts as a causative factor for the other.

2.3.1. Reactive oxygen species

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are molecules with unpaired electrons in their outer orbit. They are

generated through normal metabolic reactions and play a significant role in cellular signaling pathways (Thannickal & Fanburg, 2000). Through modulation of cellular redox state, these oxidant species are capable of inducing signaling pathways involved in gene expression, growth and differentiation, proliferation, adhesion, and apoptosis (Monteiro & Stern, 1996). The different roles of ROS in regulation of cell signaling are likely dependent on the cellular concentrations of ROS (Thannickal & Fanburg, 2000). For instance, low levels of oxidative stress in neonatal cardiac myocytes led to cell growth whereas high levels initiated apoptosis (Siwik et al., 1999).

To affect signaling pathways, ROS likely oxidize sulfhydryl groups (-SH) on critical cysteine residues of cellular proteins, modifying the activity of an enzyme if the cysteine is positioned at the catalytic site of the enzyme or modifying the ability of a transcription factor to bind to DNA if the cysteine is within its DNA binding domain (Thannickal & Fanburg, 2000). ROS can also react with thiol groups of membrane receptor proteins and change the receptor binding properties and activity (van der Vliet & Bast, 1992) or interact with DNA, leading to alterations in DNA structure (Hwang & Bowen, 2007). Many of the ROS modifications of cellular proteins and DNA are initiated from interaction of ROS with unsaturated lipids in cellular membranes (Wilhelm, 1990). ROS have also potential to interact with and damage a range of mitochondrial lipids, proteins (Paradies et al., 2001), and DNA (Lee & Wei, 2005; Kang & Hamasaki, 2003).

Superoxide ($O_2^{\cdot-}$) is the prime source of other types of ROS (Lefer & Granger, 2000). It is unstable and after formation is readily converted to hydrogen peroxide (H_2O_2) either by the enzyme superoxide dismutase or by spontaneous dismutation (Thannickal & Fanburg, 2000). Glutathione peroxidase, an enzyme located in cytosol and mitochondrial matrix (Green & O'Brien, 1970), and catalase are enzymes responsible for detoxification of H_2O_2 . Unlike catalase (Ferrari et al., 1991), glutathione peroxidase exists in considerable concentrations in the heart (Lawrence & Burk, 1978) and detoxifies most of H_2O_2 in heart mitochondria (Antunes et al., 2002).

Neither $O_2^{\cdot-}$ nor H_2O_2 have strong oxidative potential. However, H_2O_2 in the presence of reduced forms of transition metals such as iron can generate extremely reactive hydroxyl radicals ($\cdot OH$) through the so-called Fenton reaction (Thannickal & Fanburg, 2000). In biological systems, however, transition metals are sequestered by proteins such as ferritin and therefore are prevented from freely catalyzing this process.

Some of the iron captured by iron-containing proteins can be released during cardiac ischemia-reperfusion (Berenshtein et al., 1997; Coudray et al., 1994; Lipscomb et al., 1998; Voogd et al., 1992). As a result of decompartmentalization of iron, production of $\cdot\text{OH}$ increases in reperfused hearts and has been found associated with the occurrence of post-ischemic ventricular fibrillation (Tosaki et al., 1993). In this regard, iron chelators such as desferoxamine have been found advantageous in attenuating ischemia-reperfusion injury of heart (Maxwell & Lip, 1997). The mechanism of iron mobilization during ischemia-reperfusion is not yet clear. It has been found that reduced forms of flavins facilitate releasing iron from ferritin (Sirivech et al., 1974). Superoxide radicals may also contribute to this kind of release (Biemond et al., 1984). Acidosis caused by ischemia has also been proposed to stimulate iron dissociation from carrier proteins, while anoxia alone appeared insufficient to initiate iron release (Voogd et al., 1992). The last two possibilities are of importance in states of ischemia. It is worthwhile to note that although iron under conditions of oxidative stress such as ischemia-reperfusion is detrimental, at normal conditions it is harmless even though the cells are loaded with iron (van der Kraaij et al., 1988).

2.3.1.1. Sources of reactive oxygen species upon myocardial ischemia-reperfusion

During ischemia-reperfusion, production of ROS increases in the myocardium in amounts overwhelming the scavenging capacity of endogenous antioxidants (Dhalla et al., 2000). Generation of ROS increases especially within the first minutes of reperfusion and can last for hours. Bolli et al. (1988) reported the maximum production of free radicals at 2 min after reperfusion, being 90 times more than that during ischemia. The radicals then decreased after 20 min, but remained relatively elevated for a period of 2-3 h. Similarly, Duilio et al. (2001) found ROS elevation by 10 times between minutes 5 and 10 of reperfusion following 90 min coronary artery occlusion and a diminution of ROS thereafter, although ROS remained comparably high for at least 1 h. Some investigators have reported a small rise in ROS at the beginning of coronary artery occlusion as well (Zhu et al., 2007b). However, compared to coronary ligation models, hearts subjected to global ischemia seemingly generate more ROS during ischemia, probably because they establish more profound ischemia in the tissue (Zweier et al., 1987). In agreement with animal studies, lipid peroxides in coronary venous blood rose shortly after balloon inflation in 59% of patients undergoing angioplasty (Oldroyd et al., 1992).

An increasing body of evidence, mostly based on observed benefits of superoxide dismutase administration, points to $O_2^{\cdot-}$ as the primary culprit in ischemia-reperfusion injury (Marczin et al., 2003). There are many potential sources of $O_2^{\cdot-}$ including the mitochondrial electron transport system, plasma membrane and neutrophil NAD(P)H oxidase, xanthine oxidase (Sorescu & Griendling, 2002), arachidonic acid metabolism (Paravicini et al., 2004; Kamata & Hirata, 1999), cytochrome P-450, and nitric oxide synthases (Mansuy & Boucher, 2002). Mitochondria are significantly important in the heart because the heart contains a high content of mitochondria (Benard et al., 2006). However, this does not necessarily mean that cardiac cells play the major role in the production of ROS over episodes of ischemia-reperfusion, as non-myocardial cells, such as neutrophils and endothelial cells, are also of crucial importance in ischemia-reperfusion-stimulated ROS production (Figure 2.1) (Lefer & Granger, 2000). ROS generated in non-myocardial cells can diffuse to myocytes and leave detrimental effects on redox status and cellular homeostasis therein (Silverman & Stern, 1994).

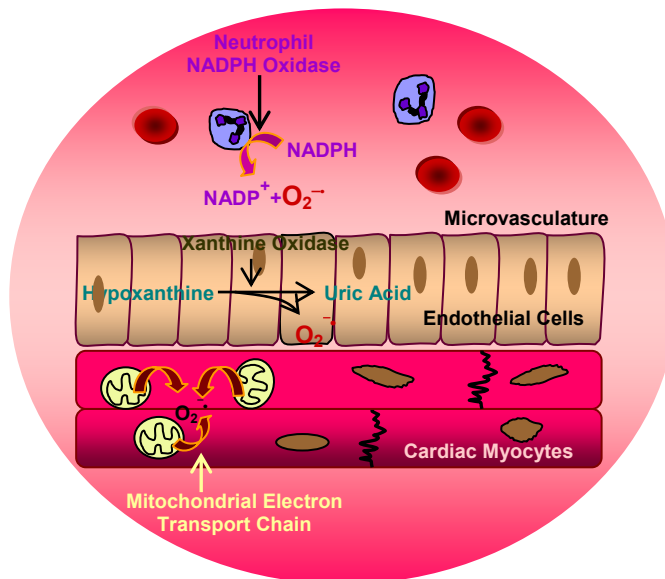


Figure 2.1. Reactive oxygen species in myocardial ischemia-reperfusion injury.

In ischemic-reperfused hearts, reactive oxygen species can be produced by various sources, particularly mitochondrial electron transport chain of cardiac cells, NADPH oxidase system of neutrophils, and xanthine oxidase enzyme of endothelial cells (adapted from Lefer & Granger, 2000).

2.3.1.1.1. Mitochondrial respiratory chain

The mitochondrial respiratory chain consumes nearly 90% of oxygen transported to the cell (Wakabayashi, 2002). Approximately 1-2% of this oxygen is converted to $O_2^{\cdot-}$ due to electron leakage from the mitochondrial electron transport chain (Kamata & Hirata, 1999) especially at the Qo site of complex III and the NADH dehydrogenase of complex I (Brookes et al., 2004) (Figure 2.2). Under normal situations, this $O_2^{\cdot-}$ causes no damage as it and the consequent H_2O_2 are quickly detoxified by mitochondrial superoxide dismutases and glutathione peroxidase, respectively (Szocs, 2004). Furthermore, cytochrome c located at the outer face of the mitochondrial inner membrane plays an important antioxidant role in the mitochondrial intermembrane space, seizing electrons from $O_2^{\cdot-}$ and transferring them to complex IV (Brookes et al., 2004). Accordingly, mitochondria not only efficiently scavenge ROS originating from intra-mitochondrial sites, but it also acts as a substantial sink for ROS from extra-mitochondrial sources (Zoccarato et al., 2004; Guidot et al., 1995).

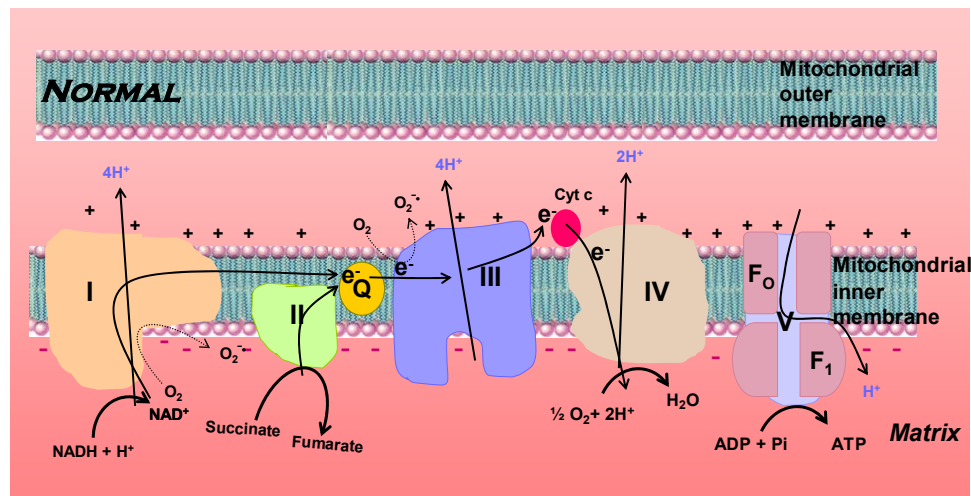


Figure 2.2. Mitochondrial respiratory chain under normal conditions.

Under physiological conditions only small amounts of electron leak from complexes I and III of the mitochondrial electron transport chain. I-V, respiratory complexes I-V; Q, coenzyme Q; Cyt c, cytochrome c; e⁻, electron.

However, mitochondria play a considerable role in production of ROS during myocardial ischemia-reperfusion (Li & Jackson, 2002; Ambrosio et al., 1993). Complexes I and III have been recognized as sites of ROS production in ischemic-reperfused hearts (Becker et al., 1999; Szocs, 2004). During ischemia or hypoxia, due to insufficient amounts of oxygen, the activity of respiratory proteins including cytochrome c oxidase (complex IV) is inhibited and the electron transport chain becomes highly reduced, giving electrons opportunity to escape from their path through respiratory complexes and to reduce the remaining oxygen with subsequent formation of $O_2^{\cdot-}$ (Szocs, 2004; Maxwell & Lip, 1997) (Figure 2.3). In contrast to this notion, Hoffman et al. (2007) reported that in isolated mitochondria ROS generation declines during hypoxia, suggesting that extra-mitochondrial sources may be involved in augmenting ROS generation upon hypoxic circumstances.

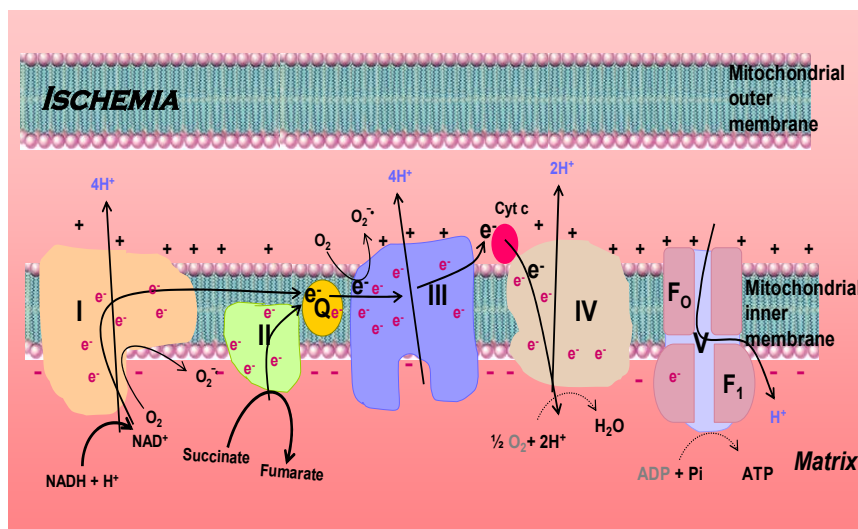


Figure 2.3. Mitochondrial respiratory chain during ischemia.

During ischemia, due to the lack of oxygen, the complex IV is inhibited, resulting in accumulation of electrons in the former complexes which results in increased superoxide production at complexes I and III. I-V, respiratory complexes I-V; Q, coenzyme Q; Cyt c, cytochrome c; e⁻, electron.

The mitochondrial production of $O_2^{\cdot-}$ also increases during reperfusion when as a result of low tissue pH the release of oxygen from hemoglobin increases (Bohr effect), producing a transient hyperoxia which leads to overactivation of respiratory chain and augmentation of electron leakage (Wolbarsht & Fridovich, 1989) (Figure 2.4). Moreover, at the beginning of reperfusion, if due to ADP shortage complex V (i.e. ATP synthase) is inhibited, electrons can leak from the prior complexes of the respiratory chain, yielding formation of more $O_2^{\cdot-}$ (Ferrari et al., 1991). Furthermore, calcium accumulation which is one of the principal events during ischemia and particularly reperfusion hastens the enzymatic reactions of the Krebs cycle in mitochondria, producing more reducing equivalents for the mitochondrial electron transport chain and enhancing production of $O_2^{\cdot-}$.

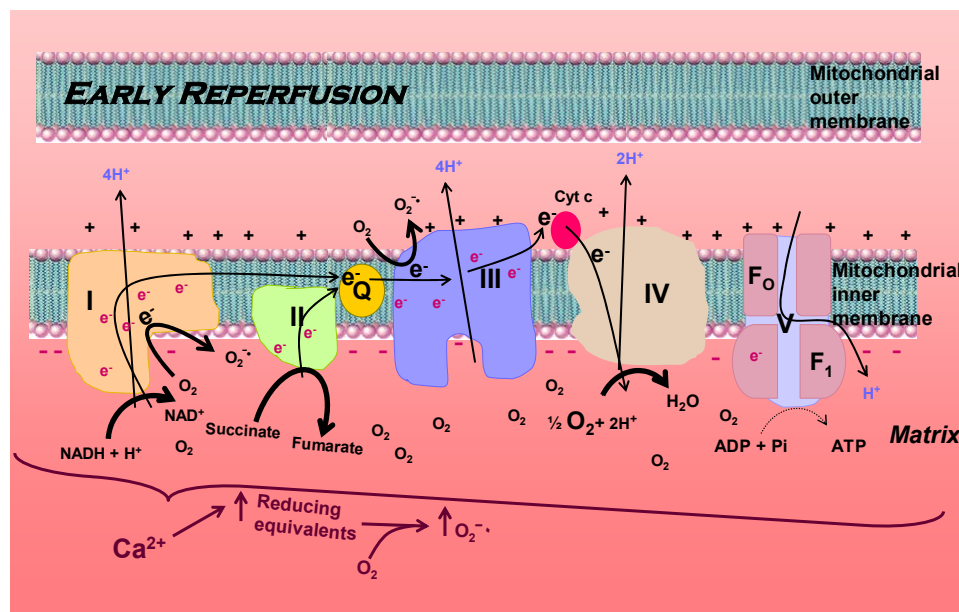


Figure 2.4. Mitochondrial respiratory chain during reperfusion.

At early reperfusion, as a consequence of increased concentration of oxygen in the mitochondrial respiratory chain and elevation of calcium in mitochondria, respiration takes place faster, and as a result, generation of superoxide increases. However, if sufficient ADP is not available, phosphorylation reactions encounter depression. I-V, respiratory complexes I-V; Q, coenzyme Q; Cyt c, cytochrome c; e^- , electron.

Reperfusion and to a lesser extent ischemia intensify formation of lipid peroxides and conjugated dienes in heart mitochondrial membrane (Petrosillo et al., 2003). Phospholipids and proteins of the mitochondrial inner membrane are the primary targets of mitochondrially generated ROS. Cardiolipin is a phospholipid in the mitochondrial inner membrane that associates with cytochrome c and other proteins of the respiratory chain, and its appearance on the outer side of the mitochondrial inner membrane is one of the earliest indices of apoptosis (Garcia et al., 2002). The structure and content of cardiolipin is crucial for well being and activity of the respiratory proteins (Petrosillo et al., 2003). However, because of its high composition of unsaturated fatty acids, cardiolipin is highly susceptible to oxidation. ROS can change cardiolipin structure and negatively impact the activity of complexes III and IV. Similarly, ischemia-reperfusion has been shown to decrease cardiolipin content in mitochondria and to reduce activities of complexes I, III and IV that could be restored by exogenous administration of cardiolipin (Petrosillo et al., 2003; Paradies et al., 2004). As a result of cardiolipin depletion in mitochondrial inner membrane, cytochrome c, an important signalling of apoptosis, can be released from the inner membrane and promote signaling pathways leading to apoptosis (Iverson & Orrenius, 2004; Lesnefsky et al., 2004b). Decreased activity of respiratory complexes due to oxidative damage following ischemia-reperfusion exacerbates electron leakage and generates more $O_2^{\cdot-}$. These perturbations can be prevented through mitigation of electron leakage from mitochondrial electron transport chain (e.g. by blockage of complex I with rotenone or amobarbitol), further indicating the significant role of mitochondria in ROS production during ischemia-reperfusion injury (Lesnefsky et al., 2004a; Aldakkak et al., 2008; Chen et al., 2007).

2.3.1.1.2. Xanthine oxidase

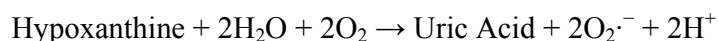
It is decades since the enzyme xanthine oxidase has been proposed as the supreme source of ROS during myocardial ischemia-reperfusion (McCord et al., 1985; Hearse et al., 1986). Recently, Duda et al. (2007) showed that allopurinol, a strong inhibitor of xanthine oxidase, almost totally blocked the generation of $O_2^{\cdot-}$ in post-ischemic guinea pig hearts. However, there is considerable skepticism about the role of xanthine oxidase in ischemia-reperfusion injury of human heart. The ambiguity exist mostly around two issues: the nature of conversion from the dehydrogenase form of the enzyme to the oxidase form during

ischemia, and more importantly is the uncertainty about the expression of the enzyme in the human heart (Nishino, 1994).

The enzyme is found primarily in its dehydrogenase form which normally consists about 90% of the total enzyme (Maxwell & Lip, 1997). This form oxidizes hypoxanthine with concomitant reduction of nicotinamide adenine dinucleotide (NAD):



A small portion of the enzyme in cells is present as the oxidase form which utilizes oxygen as electron acceptor with consequent generation of $\text{O}_2^{\cdot-}$:



Evidence shows that during ischemia some the dehydrogenase forms of the enzyme are converted to the oxidase form (Chambers et al., 1985). The conversion occurs via reversible oxidation of sulfhydryl groups or irreversible proteolytic digestion (Nishino et al., 2005). It has been suggested that the protease responsible for the irreversible conversion is calcium-dependent, raising the possibility that calcium overload caused by ischemia-reperfusion is involved in the conformational conversion of the enzyme (Maxwell & Lip, 1997). Biochemical changes happening during ischemia also provide a suitable environment for $\text{O}_2^{\cdot-}$ production by xanthine oxidase. As a consequence of blood flow cessation which limits nutrient and energy delivery to the tissue, purine nucleotides are broken down to hypoxanthine and xanthine in order to supply energy during ischemic energy deprivation. However, hypoxanthine and xanthine are substrates of the xanthine oxidase, which can begin to work and produce $\text{O}_2^{\cdot-}$ when delivery of its other substrate, oxygen, is restored to the tissue.

In heart, xanthine oxidase is mainly located in interstitial cells of the myocardium, coronary endothelial, and smooth muscle cells, but not in cardiac cells (Ashraf & Samra, 1993). The activity of xanthine oxidase in mammals varies depending on the type of tissue and the species of animal. In humans, the enzyme activity in liver and intestine is high (Parks & Granger, 1986) and in heart is negligible (Parks & Granger, 1986; Eddy et al., 1987). Nevertheless, some studies have reported protective effects of allopurinol in patients undergoing heart surgeries (e.g. Gimpel et al., 1995; Castelli et al., 1995). The reason for

this controversy may be other beneficial effects of allopurinol unrelated to its inhibition of xanthine oxidase activity. As an example, the effect of allopurinol in prevention of glutathione depletion and diminishing formation of lipid peroxides in rabbit ischemic-reperfused hearts has been suggested to be due to direct antioxidant properties of allopurinol rather than to its xanthine oxidase suppressive activity (Godin & Garnett, 1989). Despite the mentioned reasons for exclusion of a remarkable role for xanthine oxidase in heart ischemia-reperfusion injury in human, it is possible that the enzyme is responsible for at least some of the injury in human heart. Since human capillary endothelial cells contain xanthine oxidase in concentrations 1,000-10,000 times greater than other cells (Jarasch et al., 1986), it is plausible that xanthine oxidase located in the endothelium of coronary microvasculature is the origin of considerable $O_2^{\cdot-}$ in ischemic-reperfused myocardium.

2.3.1.1.3. Neutrophils

Cardiac ischemia-reperfusion injury triggers an acute inflammatory response in which neutrophils, via chemotactic attraction, infiltrate the myocardium and aggravate the damage in the already injured tissue (Frangogiannis et al., 2002). However, they are not exclusive triggers of apoptosis, as apoptosis has been observed in ischemic-reperfused myocardium even in neutropenic animals (Gottlieb et al., 1994). In their normal transit through the systemic circulation when neutrophils arrive to the reperfused tissue, they are exposed to chemotactic agents, mainly released from endothelial cells, and become activated (Korthuis & Gute, 1999) (Figure 2.5). Endothelial cells, in response to specific stimuli such as ROS (Ichikawa et al., 2004), release chemoattractants such as arachidonic acid metabolites including leukotriene B_4 and thromboxane A_2 (Welbourn et al., 1991), and adhesion molecules such as intercellular adhesion molecules (ICAM), vascular cell adhesion molecules (VCAM) and selectins, leading to neutrophil attraction, sequestration and adhesion to the microvasculature (Ichikawa et al., 2004; Seely et al., 2003). Accumulation and sequestration of neutrophils in the coronary microcirculation may lead to occlusion of the microvascular and thereby impair restoration of blood flow in the ischemic region, so-called “no-reflow phenomenon” (Schmid-Schonbein & Engler, 1986).

It is well-known that the interaction of neutrophils and endothelial cells results in endothelial cell dysfunction (Seal & Gewertz, 2005). Once activated, neutrophils release oxidants by means of their NADPH oxidase system located in their plasma membrane.

NADPH oxidase is a potential source of ROS during reperfusion; upon stimulation of the neutrophil, NADPH oxidase rapidly converts oxygen to $O_2^{\cdot-}$ (Kloner et al., 1989). Because of this powerful oxidase system, some researchers have proposed neutrophils as the major source of ROS in hearts reperfused after prolonged ischemia (Duilio et al., 2001). Furthermore, neutrophils have the enzyme myeloperoxidase, which converts H_2O_2 to the powerful oxidant hypochlorous acid. Additionally, after activation neutrophils become more adherent to the endothelium, releasing large quantities of proteases including elastase which degrade structural proteins such as elastin and cause endothelial permeability which facilitates neutrophil infiltration into the myocardium (Lefer & Lefer, 1996; Welbourn et al., 1991). When the endothelium becomes permeable, intravascular fluid can seep into the interstitial space, leading to extravascular edema and aggravating the no-reflow phenomenon (Gute et al., 1998). It is worthwhile to note that many of the mechanisms implicated in endothelial dysfunction following ischemia take place due to clearance of nitric oxide as a result of the augmented $O_2^{\cdot-}$ concentration (Szocs, 2004). We will discuss this later.

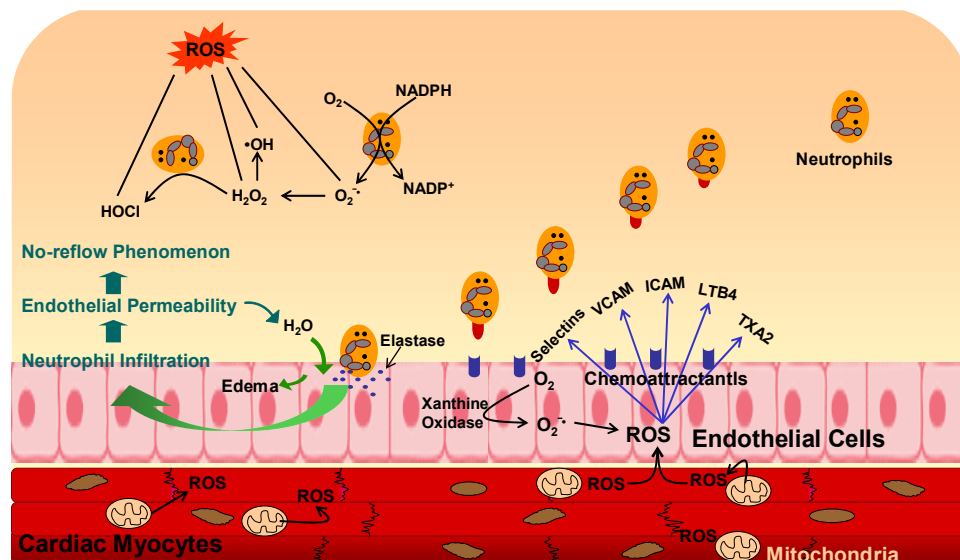


Figure 2.5. Attraction and adhesion of neutrophils to the endothelium in an ischemic-reperfused region.

As a result of the elevation of reactive oxygen species, endothelial cells release chemoattractants and adhesion molecules which make the endothelium adhesive to neutrophils. Neutrophils upon adhesion release massive amounts of oxidants and proteases that worsen the oxidative condition and permeabilize the endothelium, leading to seepage of water and electrolytes into the interstitial space beneath and development of the no-reflow phenomenon. VCAM, vascular cell adhesion molecules; ICAM, intercellular adhesion molecules; TXA2 thromboxane A_2 ; LTB4, leukotriene B_4 ; HOCl, hypochlorous acid; $\cdot OH$, hydroxyl radical.

Despite the potential of neutrophils in damaging post-ischemic myocardium, they may bring some benefits for the invaded tissue. For instance, by eliminating damaging agents and leftovers of dead cells they may attenuate intensification of inflammatory reactions (Suval et al., 1987). Furthermore, infiltrated monocytes and mast cells may contribute to healing of the injured tissue and scar formation by secreting cytokines and growth factors which are essential for proliferation of fibroblasts (Frangogiannis et al., 2002).

2.3.1.1.4. Arachidonic acid metabolites

Ischemia-reperfusion stimulates decomposition of plasma membrane phospholipids into their structural components such as arachidonic acid (Gross et al., 2005). The decomposition takes place mainly through activation of phospholipases, mostly phospholipase A₂, by ROS (van der Vliet & Bast, 1992) and/or increased levels of calcium (Ivanics et al., 2001). Arachidonic acid can be oxidized through either cyclooxygenase and lipoxygenase pathways to the corresponding products (Gross et al., 2005) or in a non-enzymatic fashion stimulated by ROS (Mobert & Becker, 1998).

Although the activity of cyclooxygenase and lipoxygenase pathways is associated with production of ROS (Gunasekar et al., 1998), there is also evidence indicating cardioprotective effects of arachidonic acid metabolites. For instance, prostacyclins which are products of cyclooxygenase are important vasodilators and therefore can be beneficial in ischemia-reperfusion circumstances (Gross et al., 2005; Mobert & Becker, 1998).

2.3.2. Calcium

Calcium overload has been shown to be a critical event in ischemia-reperfusion injury, although the exact mechanisms of the overload are still to be elucidated. Reports on the exact time of the accumulation are also rather conflicting. In general, ischemia may cause some elevation in intracellular calcium, but reperfusion is what actually causes a sharp rise in the cytosolic calcium.

Calcium overload has been suggested as a part of signaling pathways in both apoptosis and necrosis, although the degree of overload is likely higher in necrosis (Petrosillo et al., 2004). Although calcium overload by its own is a pathological stimulus possessing the capacity to initiate cellular pathways towards cell death, in most cases the switch from a

normal physiological function to a lethal effect occurs in conjunction of calcium overload with some sorts of oxidative stress (Duchen, 2000).

It is worthwhile to note that calcium in the cell behaves as a double edged sword; both calcium depletion and overload can lead to cell death. Depletion of mitochondrial calcium can lead to apoptosis due to a loss of mitochondrial membrane potential and cytochrome c release (Zhu et al., 2000). In contrast, high cytoplasmic levels of calcium can also cause increased mitochondrial calcium concentration and mitochondrial membrane depolarization, leading to either of the major forms of cell death, apoptosis or necrosis (Kruman & Mattson, 1999).

2.3.2.1. Mechanisms of calcium overload

The intracellular concentrations of calcium (Ca^{2+}) and sodium (Na^+) increase and those of magnesium and potassium decrease during myocardial ischemia-reperfusion (Shen & Jennings, 1972). The elevation in intracellular Ca^{2+} appears to be preceded by an increase in intracellular Na^+ and that in turn is preceded by augmentation in intracellular hydrogen (H^+) (Ralenkotter et al., 1997). Indeed, decline of cytosolic pH during ischemia has been shown to be the primary cause of the Ca^{2+} overload. Nevertheless, because the activity of damaging proteases and phospholipases lessens at lower pH, the acidosis may also provide benefits for the ischemic tissue (Silverman & Stern, 1994).

The mechanism of Ca^{2+} accumulation during ischemia-reperfusion as currently known is as follows: as cells face hypoxia/ischemia, they switch their means of ATP synthesis from aerobic (oxidative) to anaerobic (glycolysis) metabolism, leading ultimately to a drop in intracellular pH (acidosis) (Pierce & Czubyrt, 1995; Akabas, 2004). Increased intracellular H^+ activates cellular Na^+/H^+ exchangers to extrude H^+ ions in exchange for Na^+ , correcting the acidic pH at the expense of Na^+ accumulation. Voltage-gated Na^+ channels may also be involved in Na^+ accumulation during ischemia (Van Emous et al., 1997; Ji et al., 2004).

To get rid of the extra Na^+ , the cell has a few ways available. Cellular pathways of Na^+ efflux from the sarcolemmal membrane consist of Na^+/K^+ ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter; of these the activity of the latter is likely preserved during ischemia (Anderson et al., 1996). In contrary, due to the shortage of ATP the activity of Na^+/K^+ ATPases is suppressed during ischemia and impairs Na^+ exclusion from myocardial

cells (Cross et al., 1995). Furthermore, it has been shown that the increased concentration of inorganic phosphate (Pi) and acidic pH, both of which are characteristics of ischemia, also are important factors in the inactivation of Na^+/K^+ ATPase (Huang & Askari, 1984). Therefore, the only way that remains to expel Na^+ from the ischemic cells is the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which brings Ca^{2+} ions in while sending Na^+ out, leading ultimately to intracellular accumulation of Ca^{2+} .

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger has a crucial role in causing Ca^{2+} overload during heart ischemia-reperfusion (Akabas, 2004). The exchanger is driven electrochemically, exchanging three Na^+ ions per one Ca^{2+} . In normal myocardial function, it works in the “forward mode”, transferring Na^+ in and Ca^{2+} out of the cells. However, the mode of action could change to the “reverse mode” if the intracellular concentration of Na^+ outweighs the extracellular one, a condition that happens in ischemia-reperfusion.

Increased intracellular Ca^{2+} occurs when cells are unable to export excessive Ca^{2+} to the extracellular space. In human ventricle myocytes, the removal of Ca^{2+} from the cytoplasm is carried out mainly by the endoplasmic reticulum Ca^{2+} ATPase (70%), and partly through the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (28%) (Bers, 2002). The rest will be excluded through the sarcolemmal Ca^{2+} ATPase and mitochondrial Ca^{2+} uniporter. In ischemia, due to the lack of ATP, the activity of ATP-dependent pumps is impaired. At the same time, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is now operating in the reverse mode, carrying Ca^{2+} ions into the cytosol and taking Na^+ out. This makes the situation more troublesome and confronts cells to Ca^{2+} overload.

2.3.2.2. Mitochondria, sinks for cytosolic calcium

In cells, mitochondria are able to function as Ca^{2+} sinks, mopping up large quantities of cytosolic Ca^{2+} without harming their own normal activities (Brini, 2003; Nicholls, 2005). They act as a unique buffer for cellular Ca^{2+} , removing large amounts of Ca^{2+} from the cytosol when the intracellular concentration of Ca^{2+} is pathologically high, as in ischemia-reperfusion, and releasing it back to the cytosol but at slower speeds, enabling cells to cope with high quantities of Ca^{2+} . This is particularly important considering that intracellular concentrations of Ca^{2+} higher than normal may lead to hypercontraction of cardiac muscles. Under normal circumstances, however, the role of mitochondria in modification of cellular

Ca^{2+} is negligible as the endoplasmic reticulum has greater affinity and more rapid Ca^{2+} uptake than mitochondria (Langer, 1991; Richter & Kass, 1991).

The mitochondrial matrix is enclosed by the outer and inner mitochondrial membranes; the latter possesses a low permeability to ions (Hajnóczky et al., 2003). Ca^{2+} gains access to the intermembrane space via voltage-dependent anion channels (VDAC) located on the mitochondrial outer membrane (Brookes et al., 2004). The passage of Ca^{2+} across the inner membrane mainly occurs through the mitochondrial Ca^{2+} uniporter and this pathway actually controls Ca^{2+} uptake by mitochondria. The affinity of the uniporter for Ca^{2+} is low, and very high concentrations of Ca^{2+} are required to activate it (Brini, 2003). Exposure of mitochondria to such high Ca^{2+} concentrations is achieved through cellular localization of mitochondria in regions of high cytosolic Ca^{2+} (Duchen, 2000). Indeed, Ca^{2+} is not evenly distributed throughout the cytosol (Brini, 2003); the concentration of Ca^{2+} is higher in areas close to openings of Ca^{2+} channels of the plasma membrane and those of the endoplasmic reticulum.

Ca^{2+} is transported across the mitochondrial inner membrane using the electrochemical gradient established by the mitochondrial membrane potential ($\Delta\psi_m$) with concomitant consumption and lowering $\Delta\psi_m$ (Brookes et al., 2004). Low concentrations of Ca^{2+} in the mitochondrial matrix also provide a negative gradient for Ca^{2+} and help Ca^{2+} to enter mitochondria (Duchen, 2000). The release of Ca^{2+} from mitochondria occurs mainly via mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchangers, while the entering Na^+ is exchanged with H^+ by Na^+/H^+ exchangers, resulting in a further diminution of the membrane potential (Brookes et al., 2004). Thus, although neither Ca^{2+} influx nor Ca^{2+} efflux demands ATP, they both consume $\Delta\psi_m$ and therefore may promote depolarization of the mitochondrial membrane and collapse of $\Delta\psi_m$, thereby affecting mitochondrial function and ATP production which is critical for hearts under ischemia-reperfusion states (Duchen, 2000). Some of the Ca^{2+} may also exit from mitochondria through the permeability transition pore (PTP), which is a megachannel extended at juxtapositional sites of the two mitochondrial membranes (Brookes et al., 2004). Under physiological conditions, the PTP may transiently open to release some Ca^{2+} collected in the matrix. However, long-lasting pore opening is generally associated with pathological states.

2.3.2.3. Mitochondrial consequences of calcium overload

During ischemia, the mitochondrial uniporter probably loses its normal activity, as it relies on the mitochondrial membrane potential (Kapus et al., 1991) which also declines during ischemia (Di Lisa et al., 1995). Thus, Ca^{2+} uptake by mitochondria at the time of ischemia is largely carried out by the reverse mode of the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Griffiths et al., 1998). However, both transporters regain their normal activities at reperfusion.

In mitochondria, the primary role of Ca^{2+} is activation of oxidative phosphorylation and the major targets for Ca^{2+} are isocitrate dehydrogenase and α -ketoglutarate dehydrogenase of the Krebs cycle, pyruvate dehydrogenase, ATP synthase, and adenine nucleotide translocase (Brookes et al., 2004). In this way, Ca^{2+} enhances mitochondrial production of NADH and ATP. The overall impact of Ca^{2+} on mitochondria is acceleration of mitochondrial activities that is accompanied with further consumption of O_2 and consequently production of more $\text{O}_2^{\cdot-}$. However, the net influence of Ca^{2+} on generation of $\text{O}_2^{\cdot-}$ can not be ascertained as Ca^{2+} may in some ways decrease electron leakage from the electron transport chain. For instance, depletion of the transmembrane potential ($\Delta\psi_m$) following mitochondrial Ca^{2+} influx may decrease the electron leakage and $\text{O}_2^{\cdot-}$ production (Hajnóczky et al., 2003). Furthermore, uptake of Ca^{2+} in amounts higher than normal into mitochondria may depress respiration by inhibition of complex I activity (Hardy et al., 1991) and thereby may consequently mitigate generation of ROS.

As a result of Ca^{2+} influx into mitochondria, mitochondrial membrane potential may transiently collapse, leading to inactivation of the ATPase complex and suppression of ATP production (De Gomez-Puyou et al., 1980). Moreover, high concentrations of Ca^{2+} can activate phospholipase A_2 (Saris & Carafoli, 2005), leading to extensive conformational changes in proteins and phospholipids, particularly cardiolipin, within the mitochondrial membrane and thereby causing mitochondrial inner membrane permeability, PTP opening (Grijalba et al., 1999), and release of proteins residing in the mitochondrial intermembrane space including cytochrome c (Gogvadze et al., 2001). The opening of PTP by Ca^{2+} is considerably enhanced in the presence of ROS (Hajnóczky et al., 2003). However, if Ca^{2+} accumulation is mild, the PTP may later be resealed and mitochondria resume some of their metabolic activities to synthesize ATP required for the apoptotic death pathway, while at the same time the released cytochrome c continues its way towards activation of caspases and

initiation of the apoptotic cascade (Szalai et al., 1999). Nevertheless, even in the absence of apparent PTP opening mitochondrial Ca^{2+} accumulation alone can induce cytochrome c release (Gogvadze et al., 2001). The release of cytochrome c by Ca^{2+} could result from competition of Ca^{2+} with cytochrome c for binding to cardiolipin (Huang et al., 2006). In addition to initiation of the apoptosis cascade, the release of cytochrome c can result in inhibition of the oxidative system in the mitochondrial respiratory chain (Borutaite et al., 1999) and consequently elevation of electron leakage from the electron transport chain on one hand and blockade of ATP synthesis on the other.

2.3.2.4. Role of endoplasmic reticulum in calcium overload

The endoplasmic reticulum is the major intracellular Ca^{2+} store and plays a significant role in establishment of normal Ca^{2+} homeostasis in cells (Richter & Kass, 1991). It takes up Ca^{2+} by Ca^{2+} ATPases and releases it by two channels: the inositol 1,4,5-triphosphate (IP_3) channel and the ryanodine receptor channel; both of these channels can be activated upon elevation of intracellular concentration of Ca^{2+} (e.g. following ischemia), releasing vast amounts of Ca^{2+} into the cytosol (Boehning et al., 2003; Saris & Carafoli, 2005; White & McGeown, 2003). Also, inhibition of endoplasmic reticulum Ca^{2+} ATPase pumps, for instance as a result of ATP depletion during ischemia (Silverman & Stern, 1994), can deplete endoplasmic reticulum Ca^{2+} stores and cause a considerable rise in intracellular Ca^{2+} concentration (Bian et al., 1997). Moreover, a type of sarcolemmal Ca^{2+} entry channel (known as sarcolemmal membrane store-operated Ca^{2+} channels, among the best known of which are Ca^{2+} release-activated Ca^{2+} channels (CRAC)) is activated if Ca^{2+} stores in the endoplasmic reticulum are depleted (Hogan & Rao, 2007), further intensifying cellular Ca^{2+} overload. It is worth noting that complete depletion of endoplasmic reticulum Ca^{2+} stores can initiate apoptosis signaling independent of mitochondria via activation of Ca^{2+} -sensitive proteases (Hajnóczky et al., 2003).

The cross-talk between mitochondria and endoplasmic reticulum for maintaining cellular homeostasis and modification of pathological alterations is intriguing. The vicinity of endoplasmic reticulum and mitochondria allows this cross-talk for regulating Ca^{2+} signaling in the cell milieu (Brookes et al., 2004). For instance, as was mentioned earlier this proximity allows the uniporter in nearby mitochondria to sense high levels of Ca^{2+} and to become activated when the concentrations of Ca^{2+} in other parts of the cytosol are still not

high enough to achieve its activation (Duchen, 2000). Likewise, mitochondria keep the concentration of Ca^{2+} in areas adjacent to endoplasmic reticulum low even if the overall cytosolic level of Ca^{2+} is high, preventing stimulation of endoplasmic reticulum Ca^{2+} channels from releasing their Ca^{2+} stores into the cytosol. However, in pathological states mitochondria and eventually whole cells can be harmed from this vicinity as Ca^{2+} released from endoplasmic reticulum stores can lead to mitochondrial Ca^{2+} overload and swelling (Brookes et al., 2004).

Cytochrome c also acts as a messenger in the cross-talk between mitochondria and endoplasmic reticulum. Cytochrome c released from mitochondria by any reason (such as mitochondrial Ca^{2+} overload) can bind to the IP_3 receptor of the endoplasmic reticulum, prompting it to release Ca^{2+} (Boehning et al., 2004; Brookes et al., 2004). This creates a positive-feedback loop towards endoplasmic reticulum for emptying its Ca^{2+} stores on one side and to mitochondria for accumulating more Ca^{2+} and releasing more cytochrome c on the other.

2.3.2.5. Intracellular consequences of calcium overload

Elevation of intracellular Ca^{2+} could be detrimental for the cell in multiple ways (Silverman & Stern, 1994; Hajnoczky et al., 2003; Welbourn et al., 1991). It leads to hyperactivation of contractile proteins in heart muscle resulting in cardiac hypercontracture which is associated with increased consumption of ATP. A rise in intracellular Ca^{2+} also activates Ca^{2+} -dependent enzymes, two major classes of which are proteases and phospholipases whose activity leads to destruction of cellular constituents. For instance, increased intracellular Ca^{2+} can result in activation of calpain proteases which digest cytoskeletal and plasma membrane proteins, features that are usually observed in the necrotic form of cell death (Fink & Cookson, 2005). Furthermore, the integrity of transmembrane proteins which serve to transport ions across cellular and subcellular membranes is dependent on their surrounding phospholipids, and therefore phospholipid degradation can lead to cellular ionic derangements. On the other hand, expelling excessive intracellular Ca^{2+} is achieved mainly through ATP-dependent Ca^{2+} pumps of the endoplasmic reticulum that exhaust the available cellular ATP and leave the cell in a more problematic situation (Suzuki et al., 1997).

2.3.3. Cross-talk between ROS and calcium

ROS and Ca^{2+} do not work in completely distinct pathways. They concomitantly increase during ischemia-reperfusion and collaborate to cause the destructive biochemical consequences of the injury. Although they rise through seemingly separate mechanisms at the start, later each promotes pathological alterations in a way to intensify and aggravate the manifestations that the other one has on cellular and subcellular elements. Here are examples of possible interactions between ROS and Ca^{2+} .

Ca^{2+} overload can elevate formation of ROS. For instance, hypoxic-reoxygenated mitochondria produce more H_2O_2 when loaded with Ca^{2+} (Kowaltowski et al., 1995). Ca^{2+} can also activate Ca^{2+} -dependent proteases involved in the conversion of xanthine dehydrogenase to xanthine oxidase (Maxwell & Lip, 1997). Nitric oxide synthase is another Ca^{2+} -dependent enzyme which is activated upon Ca^{2+} overload and produces nitric oxide, a known inhibitor of respiratory chain complex IV, leading to enhanced electron leakage at complex III (Galkin et al., 2007).

Likewise, ROS can amplify Ca^{2+} overload (Suzuki et al., 1997). It is reported that both endoplasmic reticulum and mitochondrial Ca^{2+} uptake can be inhibited by oxidants, although at high concentrations of Ca^{2+} the inhibitory effect of oxidants on mitochondria is rather negligible (Kaminishi & Kako, 1989). Inhibition of Ca^{2+} uptake by endoplasmic reticulum is important as it can trigger opening of Ca^{2+} release channels and depletion of endoplasmic reticulum Ca^{2+} stores (Bian et al., 1997). In addition, ROS may oxidize critical thiol groups of ryanodine receptors of the endoplasmic reticulum, stimulating the ryanodine channels to open and release Ca^{2+} (Brookes et al., 2004). IP_3 channels also release Ca^{2+} when exposed to ROS (Suzuki et al., 1997).

Like Ca^{2+} , ROS can also facilitate PTP opening by modifying protein and lipid components of mitochondrial membranes (Hajnóczky et al., 2003). However, Ca^{2+} overload can enhance PTP opening by oxidants whereas oxidants alone are ineffective (Brookes & Darley-USmar, 2004).

2.4. Signaling pathways in cell death

2.4.1. Mitochondria: initiators or targets?

Although mitochondria have long been known as significant contributors of signaling pathways leading to cell death, recent concepts have proposed them as targets rather than initiators of cell death (Chipuk et al., 2006; Bernardi et al., 2001). It has been suggested that in response to a death stimulus mitochondria experience two events (Lutter et al., 2001). In the first stage, the outer membrane is permeabilized and some proteins situated in the intermembrane space including cytochrome c are released to the cytosol. However, the integrity of the inner membrane is preserved and mitochondria relatively maintain their normal function at this stage. In the second phase, the mitochondrial inner membrane becomes permeable, permitting ions such as Ca^{2+} and water to seep into the matrix with consequent mitochondrial depolarization, swelling, and rupture. Since in apoptosis morphological features of swelling and rupture are not seen, the second stage is more likely to occur in necrosis rather than apoptosis. In the apoptotic form of death, however, the caspase activation probably takes place during the second part of the process while initially mitochondria are protected from caspase digestion and remain functional (Von Ahsen et al., 2000). If mitochondria are able to retain their membrane potential after cytochrome c release, they can keep producing ATP to some extent before losing impermeability of the inner membrane.

How are mitochondria able to produce ATP without cytochrome c? There are two possibilities. First, a residual amount of cytochrome c may still be in the mitochondria attached to the inner membrane and keep the mitochondria operational (Von Ahsen et al., 2000). There is a report against this possibility showing that the release of cytochrome c from mitochondria in response to an apoptotic stimulus is rather rapid and coordinated, in an “all or nothing” fashion (Goldstein et al., 2000; Martinou et al., 2000). Another possibility is that small quantities of reduced cytochrome c on the cytoplasmic side of the outer membrane may act as electron carriers by entering the intermembrane space and transferring electrons from the cytosolic environment to the complex IV of the mitochondrial respiratory chain (La Piana et al., 1998). In fact, it has been shown that cytochrome c release does not hurt the integrity of the mitochondrial inner membrane, and mitochondria can sustain their activity before caspases are activated and degrade the mitochondria (Waterhouse et al.,

2001). In this way, the mitochondria can respire and maintain their membrane potential which allows them to maintain at least some of their normal activities including production of ATP, and this may even allow cells to survive mild episodes of stress.

Mitochondrial PTP opening has been suggested as the “point of no return” in mechanisms of cell death, and inhibition of PTP opening has been able to efficiently reduce cell death upon myocardial ischemia-reperfusion (Chipuk et al., 2006; Mattson & Kroemer, 2003). As was mentioned earlier, PTP consists of pores at the specific areas of the inner and outer membranes. The key components of the PTP are VDAC in the mitochondrial outer membrane, adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin D within the mitochondrial matrix (Hajnóczky et al., 2003).

The activity of the pore is regulated by a variety of stimuli. Ca^{2+} , ROS, inorganic phosphate, depletion of adenine nucleotides, disulfides, and long-chain fatty acids are among promoters of the pore opening, whereas antioxidants, high-energy phosphates, reduced glutathione, and acidic pH act as inhibitors of the pore opening (Brookes et al., 2004; Weiss et al., 2003). It is worth noting that not only oxidants promote PTP opening, but PTP also enhances ROS formation (Zorov et al., 2000; Saris & Carafoli, 2005). In fact, mitochondrial membranes act as partial barriers for respiration-produced ROS, confining ROS in mitochondria where they can be safely detoxified by mitochondrial antioxidant systems and preventing them from reaching the cytosol where they can initiate oxidative reactions. With PTP opening, this barrier no longer exists and mitochondrially generated ROS can easily access the cytoplasmic targets.

2.4.2. Cytochrome c, pro-survival and pro-apoptotic

Cytochrome c appears to have dual anti- and pro-apoptotic functions, as it participates in the production of ATP and inhibition of $\text{O}_2^{\cdot-}$ formation on one hand and in triggering apoptosis on the other. Cytochrome c can be released from cardiac mitochondria as early as 30 min after global ischemia (Borutaite et al., 2003). Also, treatment of rat embryonic ventricular cells (H9c2) with H_2O_2 has been shown to induce release of cytochrome c as early as 30 min (Han et al., 2004). It has been suggested that cells in response to apoptotic stimuli release all of their cytochrome c within 5 min (Goldstein et al., 2000). However, cytochrome c in some cell types may not be released from all mitochondria; rather it is more likely that some, but not all, mitochondria release their cytochrome c in a rapid and

coordinated fashion, quantitatively high enough to activate the apoptotic cascade (Martinou et al., 2000).

Cytochrome c is embedded into the outer face of the mitochondrial inner membrane mainly through binding to phospholipids, especially cardiolipin (Ott et al., 2002). Since the interaction of cytochrome c and cardiolipin occurs through both electrostatic and hydrophobic binding, cytochrome c may be dissociated from cardiolipin through either ionic alterations or oxidative modifications in the structure of cytochrome c and/or cardiolipin.

Cytochrome c release may occur through Ca^{2+} -dependent or Ca^{2+} -independent mechanisms (Gogvadze et al., 2001). In the Ca^{2+} -dependent mechanism, Ca^{2+} activates PTP opening leading to mitochondrial swelling and rupture followed by non-specific release of mitochondrial proteins including those in the matrix and intermembrane space to the cytosol. In the Ca^{2+} -independent mode, pro-apoptotic proteins from the Bcl-2 family especially Bax are able to induce release of cytochrome c without causing PTP opening, likely through formation of specific pores in the outer membrane (Von Ahsen et al., 2000). Bax may also enhance Ca^{2+} -dependent PTP-mediated release of cytochrome c.

The release of cytochrome c may block electron transport in the mitochondrial respiratory chain and therefore augment electron leakage at complexes I and III while mitochondria due to loss of matrix antioxidant systems following PTP probably have limited ability to efficiently scavenge the generated ROS (Brookes et al., 2004). However, this inhibition of respiration after cytochrome c release may not occur *in vivo* as mitochondria normally have plenty (20 mM) of cytochrome c, and even after losing most of their cytochrome c there may be still a sufficient amount (as little as 1 μM) to maintain respiration.

The presence of cytochrome c outside of mitochondria is likely necessary but probably not sufficient to activate apoptosis, as overexpression of Bcl-2 protein has prevented apoptosis even in the presence of intracellular cytochrome c (Gabriel et al., 2003; Zhivotovsky et al., 1998). Contrarily, cytochrome c in the cytosol may confer antioxidant activity through scavenging cytosolic $\text{O}_2^{\cdot-}$ (Brookes et al., 2004).

2.4.3. Role of the Bcl-2 family in regulation of cell death

Several proteins including those of the Bcl-2 family are believed to regulate opening of the PTP (Kutuk & Basage, 2006). Bcl-2 proteins are mainly expressed in the outer

mitochondrial membrane, the surface of the endoplasmic reticulum, and the nuclear membrane. They are categorised as anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Bcl-w, and pro-apoptotic ones, including Bax, Bak, Bad, and Bid. It has been reported that myocardial cells when subjected to an episode of ischemia-reperfusion, as an effort for survival, increase expression of Bcl-2 protein by up to 19-fold without changes in Bax expression (Kajstura et al., 1996).

Pro-apoptotic Bcl-2 family proteins have been shown to trigger release of cytochrome c and lead to apoptosis (Kutuk & Basage, 2006). Several mechanisms have been proposed for this release. For instance, pro-apoptotic Bcl-2 proteins, such as Bax and Bak, can act directly on the mitochondrial outer membrane, inducing its permeability with consequent release of cytochrome c to the cytosol (Green & Kroemer, 2004; Lutter et al., 2000; Armstrong, 2006; Hajnoczky et al., 2003). The interaction of Bax with the outer membrane may be independent of PTP opening and even happens earlier than PTP opening (Martinou et al., 2000; Green & Kroemer, 2004). The Bax-induced cytochrome c release in the absence of PTP may explain the mechanism by which cytochrome c is released from mitochondria without formation of a PTP and mitochondrial swelling. By this mechanism, cytochrome c is released in a more selective manner without changes in mitochondrial volume (Gogvadze et al., 2001).

Anti-apoptotic members of the family demonstrate effects opposite to the pro-apoptotic proteins. For instance, they protect mitochondrial outer membrane integrity and prevent release of cytochrome c (Hajnoczky et al., 2003; Armstrong, 2006; Lutter et al., 2000). They also possess antioxidant properties and attenuate oxidative stress, which is one of the factors inducing PTP opening (Hockenbery et al., 1993).

Moreover, pro- and anti-apoptotic members of the Bcl-2 family can regulate PTP opening by, respectively, increasing or decreasing sensitivity of the PTP to Ca^{2+} (Hajnoczky et al., 2003). They also affect Ca^{2+} release from endoplasmic reticulum, with anti-apoptotic members decreasing and pro-apoptotic molecules increasing Ca^{2+} release from endoplasmic reticulum (Green & Kroemer, 2004; Kruman & Mattson, 1999). In general, it is the balance between anti- and pro-apoptotic proteins of this family, such as the ratio of Bcl-2 to Bax, that determines the cell's fate of death or survival upon exposure to a pathologic stimulus.

The emerging evidence points to the existence of cross-signaling between ROS, Ca^{2+} , and Bcl-2 proteins in regulating the mitochondrial and endoplasmic reticulum response to physiological and pathological circumstances. For instance, both ROS (Buccellato et al.,

2004; von Harsdorf et al., 1999) and Ca^{2+} (Raynaud & Marcilhac, 2006; Mattson & Kroemer, 2003) can signal the activation of pro-apoptotic proteins of the Bcl-2 family. Likewise, both pro-apoptotic Bcl-2 family proteins and ROS increase Ca^{2+} release from endoplasmic reticulum (Hajnóczky et al., 2006).

2.4.4. Downstream signaling of cytochrome c

Following PTP opening, about 100 proteins are released along with cytochrome c from mitochondria, among which are apoptosis-inducing factor (AIF) and pro-caspase 9 (Martinou et al., 2000; Lopez-Neblina et al., 2005; Ott et al., 2002; Nicotera et al., 1998; Garrido et al., 2006). In the cytosol, cytochrome c in the presence of deoxy-ATP binds and activates its adaptor protein, apoptotic protease activating factor (Apaf)-1, yielding formation of an apoptosome. The apoptosome then activates pro-caspase-9 and the resultant caspase-9 in turn activates pro-caspases -3, -6, and -7, which are effector caspases for the execution of apoptosis. Since the formation of the apoptosome requires ATP, in the absence of ATP the apoptosome will not develop and the cell may switch the form of death to necrosis.

Caspases are generally divided into two groups: those which are related to apoptosis, i.e. caspase-2, -3, -6, -7, -8, -9, and -10, and those which are implicated in pyroptosis (a form of caspase-dependent cell death which is accompanied with activation of inflammatory cytokines), i.e. caspase-1, -4, -5, -11, -12, -13, and -14 (Fink & Cookson, 2005). Caspases in the first group can be divided into two subgroups: initiator caspases, i.e. caspase-2, -8, -9, and -10, and effector or executioner caspases, i.e. caspase-3, -6, and -7. The initiator caspases are firstly activated by upstream signals and then they pass the signal down by activating the effector caspases. However, the initiator caspases may also contribute to apoptosis in ways other than activation of effector caspases. For instance, caspase-9 is not only committed to activation of caspase-3, it also enlarges nuclear pores, allowing large molecules such as caspase-3 to enter the nucleus and find their targets (Faleiro & Lazebnik, 2000).

The effector caspases after activation can degrade a variety of proteins, from kinases to cytoskeletal proteins to transcriptional regulators, to develop biochemical and morphological characteristics of apoptosis (Fink & Cookson, 2005; Fan et al., 2005). Here are presented examples of their activity. Caspase-activated DNase (CAD) inhibitor (ICAD)

is one of the caspase targets which is typically bound to CAD and inhibits its activity (Fink & Cookson, 2005; Fan et al., 2005). Upon segregation from ICAD, CAD is activated and breaks DNA into certain-length fragments (Bradbury et al., 2000).

Poly (ADP-ribose) polymerase (PARP) is another target of caspases. PARP is a nuclear enzyme which mediates the repair of single-strand DNA nicks by transferring the ADP-ribose group of NAD to a number of DNA repair enzymes, recruiting them to fix the damaged DNA (Tartier et al., 2003). However, if the DNA damage is extensive, large amounts of NAD will be consumed. To replace the consumed NAD, the cell utilizes ATP, leading to ATP depletion and necrosis (Fink & Cookson, 2005). Thus, in apoptosis, although the damage to DNA as a result of fragmentation is considerable, no repair is executed as caspases digest and inactivate PARP.

Lamins and fodrin, which are important cell structural proteins, are other targets of caspases. Lamins are a family of proteins that make up the inner surface of the nucleus and are responsible for nuclear stability and chromatin structure (Ruchaud et al., 2002; Fink & Cookson, 2005; Fan et al., 2005). The cleavage of lamins by caspases causes chromatin condensation and margination, nuclear shrinkage, and DNA fragmentation. Fodrin is a major component of the membrane cytoskeleton. Breakdown of fodrin by caspases during apoptosis creates apoptotic bodies.

The enzyme transglutaminase cross-links cytosolic proteins and creates a cross-linked protein scaffold which inhibits leakage of intracellular components into the extracellular space and thus prevents provocation of inflammatory responses (Fabbi et al., 1999). In viable cells, transglutaminase exists in an inactive form which can be activated during apoptosis via cleavage by caspase-3. Transglutaminase activity leads to formation of apoptotic bodies with intact membranes.

Translocation of phosphatidylserine to the outer face of the cell membrane is another caspase-dependent hallmark of apoptosis (Botto, 2004; Wu et al., 2006). Phosphatidylserine is normally maintained on the cytoplasmic surface of the plasma membrane. During apoptosis, as a result of caspase activation, oxidative stress, and/or high intracellular Ca^{2+} , the asymmetry of phospholipids in cell membrane is lost and phosphatidylserine moves to the outer leaflet of the membrane. Phosphatidylserine exposure enables phagocytes to recognize apoptotic cells and safely engulf them before they undergo secondary necrosis with subsequent activation of inflammatory responses.

It is worthwhile to note that there is another group of cysteine proteases called calpains which are Ca^{2+} -dependent and are involved in both apoptosis and necrosis when cytosolic Ca^{2+} is elevated (Harwood et al., 2005; Fan et al., 2005; Moore et al., 2002). Calpains and caspases have common substrates. Although they can be activated by distinct stimuli and proceed through discrete pathways, they cross-talk in their signaling route towards cell death.

2.5. Cardiac performance

2.5.1. No reflow phenomenon

Reperfusion does not always lead to complete restoration of blood flow; instead some areas in the vasculature of an ischemic tissue may remain occluded or receive impaired flow. This feature of reperfusion is called the “no reflow” phenomenon and is a consequence of microvasculature plugging with neutrophils and platelets concomitant with swelling and protrusions of endothelium (Reffellmann & Kloner, 2006). Myocardial cell swelling and interstitial edema probably also contribute to the obstruction.

2.5.2. Ventricular dysfunction

Re-establishment of blood flow to an ischemic heart may not entirely restore cardiac performance (Kloner & Jennings, 2001; Ferrari et al., 1999; Rinaldi & Hall, 2000). Furthermore, an ischemic heart may never recover its normal contractility and even terminate in heart failure. There are a variety of post-ischemic myocardial contractile disarrangements. For instance, after a short non-intense ischemia the majority of cells remain alive but the heart may not regain its normal contractility for a variable course of time lasting from hours to days. This transient reversible contractile depression is termed “stunning” and occurs despite restoration of the blood flow to the pre-ischemic level. On the other hand, a chronic mild ischemia can result in impaired blood flow concomitant with chronic depressed contractile function, called “hibernating”. However, as the damage is reversible, restoration of flow can lead to recovery of contractility.

Cardiac arrhythmia is another type of perturbation after ischemia, and can be associated with manifestations of ventricular tachycardia and fibrillation (Maxwell & Lip,

1997). Reduction in systolic pressure and augmentation in diastolic tension are among the hemodynamic disturbances of reperfusion (Lefer & Granger, 2000). Since ROS and Ca^{2+} are blamed as principal factors causing ischemia-reperfusion damage, antioxidants and inhibitors of Ca^{2+} overload may be effective interventions for alleviating contractile disturbances after ischemia (Bolli, 1994 & 2003).

2.5.3. Contribution of energy depletion to cardiac contractile dysfunction

As heart contractility is highly dependent on cellular ATP resources, impairment of the mitochondrial oxidative phosphorylation system has a tremendous impact on the heart well being and function (Brookes et al., 2004). During ischemia, cells that are deprived of oxygen switch their metabolism from aerobic to anaerobic, and rely on the limited amount of ATP that is produced through glycolysis. This leads to decline of intracellular pH on one hand and rapid ATP depletion on the other. Depletion of cellular ATP is associated with ischemic contracture, also known as rigor contracture or rigor shortening (King & Opie, 1998; Kingsley et al., 1991). The contracture is caused by the strong interaction between actin and myosin that in experimental conditions occurs at very low ATP concentrations. The development of rigor contracture is a key event happening during the progress of cell injury. It is worthwhile to note that excessive concentrations of Ca^{2+} at the beginning of reperfusion concomitant with restoration of ATP supply and normalization of intracellular pH may also trigger hypercontracture, although this time upon reperfusion (Piper et al., 1998).

2.6. Endothelial dysfunction

As a result of ischemia-reperfusion, the cardiac circulatory system loses its normal function mainly due to endothelial dysfunction (Szocs, 2004). Endothelial function is dependent on the cellular level of nitric oxide (NO). The production of NO in endothelial cells drops as early as 2.5-5 min following the onset of reperfusion (Lefer & Lefer, 1996) and emerging evidence indicates that stimulation of endogenous NO production or administration of NO substrate improves resistance of hearts to ischemia-reperfusion injury, while inhibition of NO generation worsens post-ischemic complications (e.g. Liu et al., 2007a; Pozo-Navas et al., 2006; Li et al., 2006b). NO has been recognized as the trigger as

well as a mediator of ischemia preconditioning (Dawn & Bolli, 2002; Szigeti et al., 2004; Li et al., 2004).

NO has vasodilatory, anti-inflammatory, and anti-platelet activities (Laursen et al., 2006; Reichenbach et al., 2005). It is unstable, possessing a half-life as short as 1 s (Miller, 2001; Szocs, 2004). The NO half-life in biological environments closely depends on the concentration of $O_2^{\cdot-}$. Antioxidants, such as superoxide dismutase, glutathione, and vitamins E and C, have been shown to elevate NO bioavailability.

Several mechanisms have been proposed for NO protection of myocardium from ischemia-reperfusion injury. For instance, NO prevents neutrophil adhesion to the endothelium via reducing expression of endothelial adhesive molecules such as ICAM-1 and P-selectin, therefore inhibiting neutrophil and platelet aggregation and activation and suppressing subsequent inflammation (Pagliaro et al., 2003; Laude et al., 2002).

In contrary, injurious effects of NO have also been reported. These effects are the outcome of the reactivity of NO with other reactive molecules. NO is typically a low activity ROS that can react with other free radicals such as superoxide and lipid peroxyl radicals. The products, such as peroxynitrite, react with amino acid residues on proteins, leading for instance to modification of cysteine residues (S-nitrosylation) in proteins and thereby altering the conformation and activity of cellular enzymes such as those involved in the mitochondrial respiratory chain (Clementi et al., 1998). Most of detrimental effects of NO are attributed to peroxynitrite (Pacher et al., 2007; Ferdinandy & Schulz, 2003).

Peroxynitrite ($ONOO^-$), a product of reaction of NO with $O_2^{\cdot-}$, can decompose to strong oxidizing agents such as nitrogen dioxide and hydroxyl radical that can nitrate and hydroxylate cellular biomolecules including lipids, proteins, and sugars (O'Donnell et al., 1999). Although hydroxyl radicals have been designated as the most toxic species of ROS, $ONOO^-$ may be even more destructive as it is comparably more stable and therefore can diffuse farther into cellular compartments and even pass the cell membrane and hit extracellular targets (Beckman et al., 1994).

Marked increases in production of $O_2^{\cdot-}$ during ischemia-reperfusion provide a suitable context for formation of $ONOO^-$. Growing evidence indicates that levels of NO, $O_2^{\cdot-}$, and $ONOO^-$ greatly increase at reperfusion, and that application of NOS inhibitors or superoxide dismutase has beneficial impacts on contractile performance of hearts after ischemia (Pacher et al., 2007). Mitochondria, being the primary cellular producers of $O_2^{\cdot-}$, have been suggested as a great source of $ONOO^-$ generation (Packer et al., 1996). The produced

ONOO⁻ can damage mitochondrial proteins, lipids, and DNA. As a target, ONOO⁻ can inactivate manganese superoxide dismutase (MnSOD), one of the two chief mitochondrial O₂⁻ quenchers (Macmillan-Crow & Cruthirds, 2001). Indeed, ONOO⁻ appears to be the only oxidant capable of inactivating MnSOD through nitration of critical tyrosine residues. It is noteworthy that in the presence of physiological concentrations of ascorbate, mitochondria detoxify peroxynitrite via its decomposition to yield NO (Barone et al., 2003).

NO is produced by nitric oxide synthases (NOS). Three isoforms of NOS have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Activation of nNOS and eNOS has been found necessary and advantageous for normal function of endothelium, while induction of iNOS results in generation of large amounts of NO which may have deleterious effects (Razavi et al., 2005). All three NOS isoforms are expressed in cardiac myocytes (Raij, 2006). In biphasic fashion, myocardium uses eNOS and iNOS for rapid and prolonged production of NO, respectively. During reperfusion, production of moderate amounts of NO (probably by eNOS) inhibits neutrophil infiltration and results in myocardial protection (Vinten-Johansen et al., 1999). However, depending on the amount of oxidative stress (Berges et al., 2003) high levels of NO produced by iNOS may deliver protective (Wakeno-Takahashi, 2005; Das & Sarkar, 2007) or harmful effects (Li et al., 2005; Egi et al., 2004).

Depending on pathological circumstances and concentration of NO, NO may display anti- or pro- apoptotic properties (Razavi et al., 2005). Anti-apoptotic effects of NO are mainly mediated by low amounts of NO following stimulation of eNOS. The eNOS has shown the potential to block apoptosis in myocardial infarction and heart failure. The anti-apoptotic effect of NO is performed through S-nitrosylation of proteins that are implicated in apoptosis signaling, such as caspases, activator protein-1, and transglutaminase. Some of the protective effects of NO against apoptosis may also be exerted through induction of protective proteins such as heme oxygenase-1, heat shock proteins, and metallothionein. The pro-apoptotic effects of NO are mostly due to high concentrations of NO produced by iNOS, resulting in the generation of ONOO⁻. However, ONOO⁻ alone may not be sufficient to develop PTP opening and apoptosis, as it particularly promotes PTP in Ca²⁺-loaded mitochondria, pointing to a synergism between Ca²⁺ and ONOO⁻ and exemplifying the extent of complications in ischemia-reperfusion injury (Brookes & Darley-USmar, 2004). Moreover, high levels of NO may switch the form of cell death from apoptosis to necrosis

by inhibiting mitochondrial respiration and decreasing ATP synthesis in spite of deferring cytochrome c release and inactivating the effector caspases (Leist et al., 1999).

2.7. Ischemia preconditioning

Ischemia preconditioning is one of the most potent anti-ischemic interventions known to date. It consists of increasing resistance of an organ, tissue, or a number of cells by means of utilizing a non-harmful stimulus prior to the exposure to a noxious insult such as ischemia (Bolli, 2007; Das & Maulik, 2006). Preconditioning may be exerted by a wide variety of stress stimuli; one of the most investigated is achieved by brief (3-5 minutes) transient cycles of ischemia and reperfusion. Although the mechanisms of preconditioning are not entirely elucidated, it is likely that in response to mild primary instigations specific proteins in cells are activated and particular genes are expressed, leading ultimately to reinforcement of the tissue for better withstanding subsequent stresses. In other words, upon preconditioning the heart senses the stress and changes its phenotype in a way to be more defensive and better protected.

Preconditioning consists of two phases: an early phase (classical preconditioning), which appears within minutes and wanes within 2-4 hours, and a late phase, which occurs 12-24 hours later and persists for 3-4 days (Bolli, 2007; Eisen et al., 2004; Qiu et al., 1997) (Figure 2.6). The mechanisms of cardioprotection by the two phases are probably different; while post-translational alterations such as changes in the activity of existing proteins are suggested to develop the early phase, induction and expression of relevant genes and synthesis of proteins is probably the basis of the late phase. The cardioprotective strength of the two phases is also different. The early phase is highly powerful but short-lasting, whereas the late phase is less effective but lasts longer. Therefore, the early phase alleviates the severity of damage (infarct size) in the acute phase and reduces mortality, while the delayed type protects mainly against the chronic aspects of injury such as contractile dysfunction (e.g. myocardial stunning) although it is also effective in reducing infarct size.

Still much needs to be known about preconditioning and its underlying mechanisms. The early preconditioning relies on recruitment of G-protein-coupled receptors to activate a number of signaling pathways including various protein kinases and eNOS (Murphy, 2004; Post & Heusch, 2002; Otani, 2004) (Figure 2.6). These signaling pathways cardioprotect through effects on mitochondria, the best known effect being activation of mitochondrial

K_{ATP} channels. Mitochondrial K_{ATP} channels, which activate an inward current of potassium across the mitochondrial inner membrane, reduce the membrane potential and therefore attenuate mitochondrial Ca²⁺ uptake. Mitochondrial K_{ATP} channels may also increase Ca²⁺ release in mitochondria. Additionally, the opening of mitochondrial K_{ATP} channels results in a mild increase in mitochondrial volume (swelling) that causes a trend towards closing the voltage-dependent anion channels (VDAC). Closing such channels is beneficial as it prevents transmission of glycolytically-generated ATP from cytosol to the mitochondria, where it can be consumed by F₁F₀-ATPase activity favoured during ischemia as a result of decreased membrane potential.

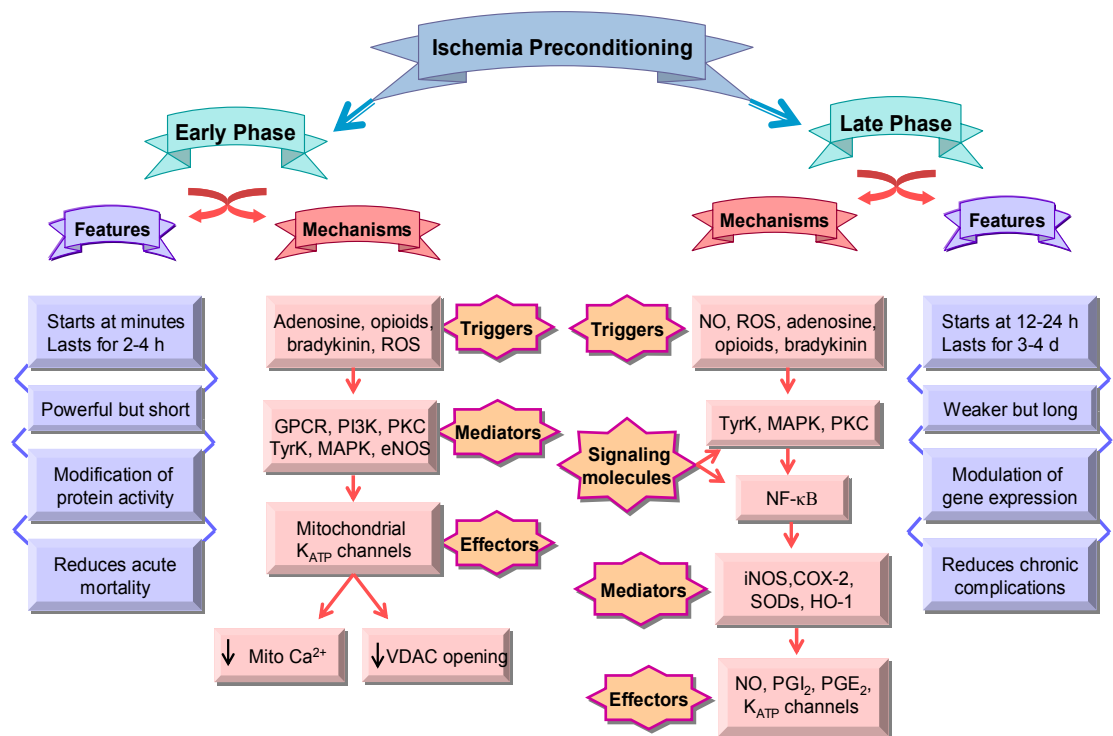


Figure 2.6. Major differences in the characteristics and proposed mechanisms of ischemia preconditioning.

ROS, reactive oxygen species; GPCR, G protein-coupled receptor; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; TyrK, tyrosine kinase; MAPK, mitogen-activated protein kinases; eNOS, endothelial nitric oxide synthase; Mito, mitochondria, VDAC, voltage-dependent anion channels; NO, nitric oxide; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; SODs, superoxide dismutases; HO-1, heme oxygenase-1; PGI₂, prostacyclin; PGE₂, prostaglandin E₂.

The signaling pathways evoked by early preconditioning also trigger transduction pathways for development of late preconditioning. Transduction pathways that are activated during the process of late preconditioning lead to activation of transcription factors (especially nuclear factor- κ B) and subsequently induction of corresponding genes and protein synthesis (Bolli, 2007; Post & Heusch, 2002; Otani, 2004). Proteins such as iNOS, cyclooxygenase-2, heme oxygenase-1, and superoxide dismutases have been recognized as mediators of late preconditioning. iNOS and cyclooxygenase-2 probably work through improvement of vascular flow, and superoxide dismutases and heme oxygenase-1 act mainly via suppression of ROS. Elevation of activity and/or expression of endogenous antioxidants have been shown following ischemia preconditioning (Dhalla et al., 2000). However, the absence of augmented levels of antioxidants and antioxidant enzymes has also been reported in preconditioned myocardium by a number of investigators (Tang et al., 1997), although the induction of ischemia preconditioning was not verified in their study.

Late preconditioning or so-called “second window of protection” is more beneficial than the early phase as it produces prolonged protection. The late preconditioning can be developed by various stimuli, dividable into two groups: non-pharmacological, such as ischemia and exercise, and pharmacological, such as adenosine agonists and NO donors (Eisen et al., 2004). ROS can also act as triggers of the late preconditioning. Administration of a complex of ROS scavengers has been shown to block activation of this type of preconditioning (Sun et al., 1996). Nevertheless, ROS are advantageous as effectors of the late preconditioning as long as their cellular levels do not exceed the antioxidant capacity of the cell; otherwise they will be more destructive than helpful.

Preconditioning of the myocardium has been revealed to decrease myocardial energy demand, resulting in a reduced rate of ATP consumption and thereby a diminished rate of anaerobic glycolysis and lactate production, which in turn prevents acidosis (Eisen et al., 2004) and accumulation of Na^+ and Ca^{2+} , resulting ultimately in diminution of overall injury.

Recently, a protection by similar repetitive brief cycles of ischemia and reperfusion applied at the beginning of reperfusion, called post-conditioning, has been discovered (Vinten-Johansen, 2007; Rodríguez-Sinovas et al., 2007). Post-conditioning is in fact a form of gradual reperfusion which has long been known to be effective to prevent reperfusion injury. The mechanism of protection by post-conditioning is unclear, but it seems that

deceleration of neutralizing pH and gradual reintroduction of oxygen which allow cells to prevail over ionic and oxidative disturbances at early reperfusion are implicated.

2.8. Cellular antioxidant network

2.8.1. Non-enzymatic antioxidants

Human studies have shown depletion of non-enzymatic antioxidants such as glutathione, ascorbic acid, and vitamin E following myocardial ischemia-reperfusion (Marczin et al., 2003). Hydrophilic antioxidants, such as ascorbate and glutathione, that work in the cytosol and extracellular fluids seem to respond to oxidative stress earlier than lipophilic antioxidants such as ubiquinol and vitamin E that act in membranes (Haramaki et al., 1998). This suggests that antioxidants in cells are engaged in a network, having a systematic relationship with one another and being utilized in a certain order. In such a network, aqueous phase antioxidants, e.g. ascorbate and glutathione, work at the front line of defence against oxidative stress, protecting aqueous cell biomolecules such as proteins and DNA from oxidation, and also helping to regenerate lipid phase antioxidants from their oxidized forms. In this system, ascorbic acid serves to regenerate vitamin E from vitamin E radicals (Nagaoka et al., 2007), and is in turn regenerated by glutathione (May et al. 1996). Therefore, lipid phase antioxidants are likely preserved in cells as long as hydrophilic antioxidants have not been consumed.

Many authors have pointed to the existence of the antioxidant network in the cellular entity (Marczin et al., 2003; Blomhoff, 2005). This antioxidant network concept authenticates the preference of combined treatments of antioxidants over their separate application upon conditions of severe oxidative stress such as ischemia-reperfusion.

2.8.1.1. Glutathione

Glutathione (GSH) or γ -glutamyl-cysteinyl-glycine is the prominent intracellular non-protein sulfhydryl compound that is found in cells in millimolar (0.5-10) concentrations (Lash, 2006; Chakravarthi et al., 2006; Wu et al., 2004; Dickinson & Forman, 2002). GSH is mainly synthesized in the liver, although it can be made in any cell type. Since cytoplasm is the major site of GSH synthesis, most of cellular GSH (85-90%) is found in the cytosol,

with the rest being located mainly in mitochondria, nuclear matrix, and peroxisomes. The activity of GSH relies on the thiol residue of the cysteinyl group that allows glutathione to act as both a reductant and a nucleophilic compound.

By working as a reductant, GSH can react in a direct non-enzymatic mode with carbon-centred radicals, donating an electron from its cysteine residue and being itself oxidized to glutathione disulfide (GSSG) (Lash, 2006). Moreover, GSH keeps sulfhydryl residues of cellular proteins in their reduced form. This protection of sulfhydryl groups by GSH could happen through reducing highly reactive peroxides catalyzed by glutathione peroxidase or through entering in thiol-disulfide exchange reactions catalyzed by thiol transferases. As a nucleophile, GSH is conjugated to electrophilic groups of xenobiotic and cytotoxic compounds by means of the catalytic effect of glutathione S-transferases, making hydrophilic glutathionyl conjugates from toxic substances that can then easily be excreted. Thus, the major role of GSH is to maintain intracellular redox homeostasis and to defend cells not only against ROS but also against other toxic insults.

The conversion of intracellular GSSG to GSH is performed by glutathione reductase, a flavoprotein that converts one molecule of GSSG to two molecules of GSH using NADPH as electron donor (Noctor et al., 2002). Thus, the amount of GSH + 2GSSG defines total cellular glutathione, almost 15% of which is attached to proteins (Wu et al., 2004). The ratio of GSH to GSSG reflects cellular redox state and antioxidant potential of cells and is generally more than 10 under normal circumstances. Apart from conversion to GSH, an alternative route for intracellular GSSG is exclusion from cells, a mechanism that prevents suppression of the cellular GSH to GSSG ratio.

The extent of myocardial damage and the speed of mechanical recovery after ischemia-reperfusion are strongly dependent on glutathione levels in the heart tissue (Singh et al., 1989). Accordingly, DNA fragmentation that occurs during the process of cell death has been found associated with oxidative stress-induced glutathione depletion (Higuchi, 2004). Administration of glutathione has mitigated post-ischemic injury in rat hearts where ascorbic acid was ineffective, although the best protection was observed in co-treatment with glutathione and ascorbate, implying the existence of a synergism between the two hydrophilic antioxidants (Gao et al., 2002).

Ischemia-reperfusion has been demonstrated to result in a decrease in glutathione and an increase in malondialdehyde in hearts of animals (Ozer et al., 2005; Lapenna et al., 1996; Roth et al., 1997) and heart (De Vecchi et al., 1998) and blood samples (Akila et al., 2007)

of humans. Lowered concentrations of the reduced form of glutathione are likely to be concurrent with elevated levels of its oxidized form (Ji et al., 1993; Arduini et al., 1988). Alteration in glutathione status of the myocardium is supposedly worsened by intensification of oxidative stress during ischemia-reperfusion (Haramaki et al., 1998). Reperfusion has been shown to cause a sustained liberation of the reduced and especially oxidized forms of glutathione from the heart concomitant with suppressed cardiac function, both correlating with the duration of ischemia (Ferrari et al., 1990).

Glutathione is one of the few agents which detoxify peroxynitrite without forming other toxic compounds (Ronson et al., 1999). Hearts treated with glutathione had a reduced formation of peroxynitrite in the first minutes of reperfusion and better mechanical function after ischemia and upon exposure to peroxynitrite (Cheung et al., 2000). The reaction of glutathione with peroxynitrite results in formation of thiol-containing compounds such as nitrosothiols that can subsequently convert to nitric oxide (Ronson et al., 1999). Furthermore, biological properties of nitric oxide and nitrosothiols are similar. For instance, similar to nitric oxide, nitrosothiols have vasodilatory and anti-neutrophil activities (Rassaf et al., 2002; Ronson et al., 1999).

2.8.1.2. Ascorbic acid

Plasma levels of ascorbate decrease upon ischemia-reperfusion in human hearts and may remain low for up to 2 weeks (Ballmer et al., 1994). Similarly, tissue concentrations of ascorbate diminish in ischemic-reperfused hearts depending on the length of reperfusion and severity of oxidative stress (Haramaki et al., 1998). The reduction in cardiac tissue ascorbate is associated with release of ascorbyl radicals in coronary flow (Pietri et al., 1990). Nevertheless, oral supplementation with vitamin C may or may not lead to alleviation of the injury. For instance, whereas in a study high dosage of ascorbic acid exhibited a remarkable protection on myocardium of patients undergoing bypass operation (Dingchao et al., 1994), in another study application of high-dose ascorbic acid, although it increased total antioxidant capacity and preserved antioxidant enzyme activities in plasma of patients undergoing heart valvular surgery, it did not prevent metabolic alterations such as increased generation of lactate during ischemia (Marczin et al., 2003). Similarly, some animal studies have also found no cardioprotection from ascorbate against ischemia-reperfusion injury

(Rump et al., 1998). The difference in experimental conditions such as dosage and time of ascorbate administration may be the cause of the discrepancies.

The effectiveness of ascorbate administration in attenuation of myocardial microvascular damage after ischemia has been found greater than that of Trolox, although the best protection was seen in application of the combined treatment (Molyneux et al., 2002). Ascorbate also has a sparing effect on glutathione (Meister, 1994).

2.8.1.3. Vitamin E

Similar to other antioxidants, myocardial tissue vitamin E level depletes during ischemia-reperfusion events (Barsacchi et al., 1992; Coghlan et al., 1993; Westhuyzen et al., 1997). Likewise, a drop in blood levels of α -tocopherol and β -carotene after myocardial infarction in humans has also been observed in some (Muzakova et al., 2001) but not all studies (Ballmer et al., 1994; Tangney et al., 1998). Similar to ascorbic acid, vitamin E supplementation may or may not deliver beneficial effects. The disagreements may be a result of difference in severity of oxidative stress, the status of other antioxidants, and the time course of vitamin E supplementation. For instance, vitamin E may be more helpful in severe cases of myocardial infarction (Dhalla et al., 2000). Further, studies show that the increase of vitamin E concentration in myocardial tissue after oral administration may require days to weeks (Mickle et al., 1991). However, even if an appropriate amount of vitamin E is loaded in the tissue and prevents vitamin E depletion during cardiac operation, expected biochemical and clinical benefits may not be achieved (Westhuyzen et al., 1997).

2.8.2. Enzymatic antioxidants

ROS are formed during normal cellular metabolism and under pathophysiological circumstances. To quell deleterious effects of ROS, cells have developed an organized system of antioxidant enzymes. They engage, for instance, superoxide dismutase to detoxify $O_2^{\cdot-}$ with subsequent production of H_2O_2 , which is in turn converted to water by glutathione peroxidase or catalase with simultaneous oxidation of glutathione (Dhalla et al., 2000; Dickinson & Forman, 2002). The oxidized glutathione can then be reduced back to the glutathione by glutathione reductase. Glutathione S-transferase is another enzyme which conjugates electrophiles and lipid peroxides with glutathione, facilitating their excretion

from the body. Therefore, similar to non-enzymatic antioxidants, the enzymatic antioxidants participate coordinately in reactions against oxidative stress. As depicted in the Figure 2.7, the coordinated action of various cellular antioxidants ensures efficient detoxification of ROS and electrophilic compounds.

Although the available literature mostly indicates depletion of non-enzymatic antioxidants during ischemia-reperfusion, reports on activity of enzymatic antioxidants are contradictory as all cases of increase, decrease, or no alteration in their activities have been reported (Marczin et al., 2003; Arduini et al., 1988). For instance, some studies have shown no effect of ischemia-reperfusion on activities of catalase, glutathione peroxidase, and superoxide dismutase (Kihlstrom et al., 1989; Coudray et al., 1995), or on glutathione

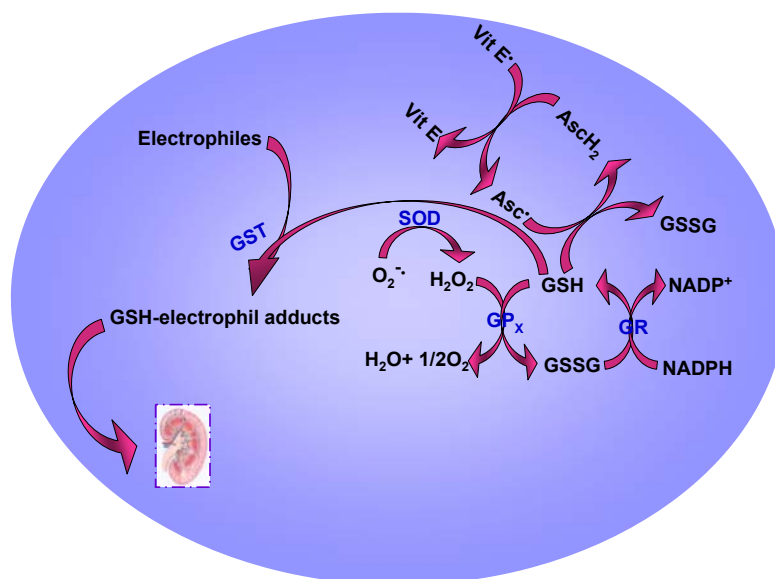


Figure 2.7. Coordinated action of some of cellular antioxidants.

GSH, glutathione; GSSG, glutathione disulfide; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; SOD, superoxide dismutase; AscH₂, ascorbic acid; Vit E, vitamin E; Vit E[•], vitamin E radical (adapted from Juurlink, 2001).

peroxidase and glutathione reductase (Curello et al., 1985). In contrast, some investigators have reported reduced activities of glutathione peroxidase, glutathione reductase, and catalase (Ji et al., 1993), reduced activities of glutathione peroxidase and catalase and increased activity of glutathione S-transferase (Porreca et al., 1994), or decreased activity of glutamate cysteine ligase and unmodified activity of glutathione reductase (Lapenna et al., 1996). Similar discrepancies have been observed in human studies. For instance, cyanotic patients undergoing open heart surgery showed elevated activities of glutathione peroxidase, catalase, and superoxide dismutase after reperfusion (Kim et al., 1994), whereas patients undergoing cardiopulmonary bypass had lower catalase, higher glutathione reductase, and unchanged glutathione peroxidase activities after the surgery relative to the pre-operation values (Inal et al., 1999).

Also, in some reports antioxidant enzyme activities have changed during ischemia but returned to the pre-ischemic levels upon reperfusion. For instance, in a study by Prasad et al. (1992) activities of superoxide dismutase and catalase decreased during ischemia and returned to control levels during reperfusion. A similar pattern was observed after increased activity of glutathione reductase during ischemia (Arduini et al., 1988). Moreover, the response of enzyme activities to stimuli may differ depending on the isoform of the enzyme and its cellular location. For example, the mitochondrial form of superoxide dismutase decreased after both ischemia and reperfusion, whereas the cytoplasmic type remained unchanged (Arduini et al., 1988). Likewise, although the activities of both cytoplasmic and mitochondrial glutathione peroxidase increased upon ischemia, only the mitochondrial form showed further elevation during reperfusion.

The activity of catalase in the myocardium is known to be scant (Dhalla et al., 2000). Nonetheless, catalase might have a significant role in protecting hearts during ischemia-reperfusion where the concentration of ROS in myocardium increases remarkably. However, results of administration of catalase as well as that of superoxide dismutase are inconclusive. For example, although both superoxide dismutase and catalase reduced production of hydroxyl radicals in ischemic-reperfused hearts, they conferred no improvement in cardiac contractile function (Tosaki et al., 1993). Combined treatment with superoxide dismutase and catalase, however, was effective in improving post-ischemic myocardial function (Kazimoglu et al., 2004; Tosaki et al., 1993; Galang et al., 2000; Lefer & Granger, 2000). Nevertheless, there are also studies that have not found improvement of contractile function

and limitation of infarct area upon co-administration of superoxide dismutase and catalase (Przyklenk & Kloner, 1989).

2.8.2.1. Phase 2 enzymes

Xenobiotics and antioxidants in cells provoke coordinated induction of a particular class of genes which enhance cellular defence against oxidative stress (Jaiswal, 2004; Chen & Kunsch, 2004; Lee & Johnson, 2004). These genes encode for a number of phase 2 detoxification enzymes including NAD(P)H:quinone oxidoreductase (NQO)1, NQO2, glutathione S-transferase, γ -glutamate cysteine ligase (GCL), heme oxygenase (HO)-1, UDP glucuronyl transferase, ferritin, and metallothionein-1. Promoters of these genes contain a common *cis*-element, termed antioxidant response element (ARE), which is involved in the transcriptional activation of the genes primarily governed by nuclear factor-E2-related factor-2 (Nrf2). Over exposure to oxidative stress the expression of genes possessing ARE is coordinately regulated by Nrf2. Thus, Nrf2-deficient mice are very sensitive to electrophilic and chemical damages. Nrf2 is normally sequestered in the cytosol by Keap1 (Kelch-like ECH-associated protein 1) (Jaiswal, 2004). The mechanism of Nrf2 activation is not yet clear. Phosphorylation of Nrf2 and modification of redox-sensitive cysteine residues in Keap1 are among the possible ways to dissociate Nrf2 from Keap1.

A number of antioxidant enzymes such as superoxide dismutase and catalase are not currently known as phase 2 enzymes; however, they may be induced by Nrf2. Zhu et al. (2005) showed that incubation of cardiac fibroblasts by dithiole-3-thione led to induction of Cu/Zn-superoxide dismutase, catalase, glutathione, glutathione reductase, glutathione peroxidase, glutathione S-transferase, and NAD(P)H:quinone oxidoreductase; the expression of all except glutathione peroxidase was abolished in Nrf2-/- cells. Similarly, Chen and Kunsch (2004) have listed a number of detoxification and antioxidant genes that their coordinated expression is regulated through an Nrf2/ARE mechanism; among these genes are glutathione-dependent genes, such as glutathione peroxidase, glutathione reductase, peroxiredoxin, as well as two well known phase 2 enzymes glutathione S-transferase and glutamate cysteine ligase. It is noteworthy that phase 2 enzymes may be induced by transcription factors other than Nrf2. For instance, HO-1 may be induced by Nrf2 as well as activator protein-1 (AP-1) and NF- κ B (Hill-Kapturczak et al., 2001). Similarly, AP-1, NF-

κ B, Nrf1, and Nrf2 mediate induction of glutamate cysteine ligase (Soltaninassab et al., 2000; Kwong et al., 1999).

2.8.2.1.1. NAD(P)H:quinone oxidoreductase

NAD(P)H:quinone oxidoreductase (NQO1), also referred to as DT-diaphorase, is a flavoprotein that, using either NADH or NADPH as electron donor, catalyzes the two electron reduction of electrophilic compounds quinones and quinonoids to hydroquinones without formation of semiquinones (Ames et al., 2002; Chen et al., 2000b; Ross, 2004; Chen & Kunsch, 2004). The hydroquinones then can be conjugated and excreted. The two electron reduction is a unique feature of NQO1 as other quinone reductases such as cytochrome P450 reductase catalyze one electron reduction to generate semiquinone radicals which can stimulate oxidative reactions. Interestingly, NQO1 has long been recognized as a chemoprotective agent and its induction is used as a means for identification of potential anti-carcinogens (Dinkova-Kostova & Talalay, 2000).

NQO1 is mainly committed to maintain two critical lipid soluble antioxidants, coenzyme Q and vitamin E quinone, in their reduced forms, i.e. ubiquinol and vitamin E hydroquinone, thereby protecting cellular membranes from oxidative damage (Genova et al., 2003; Dinkova-Kostova & Talalay, 2000). NQO1 has also been recognized as an $O_2^{\cdot-}$ scavenger, although weaker than superoxide dismutase (Zhu et al., 2007a). However, the high levels of NQO1 in cells especially after exposure to oxidants make it a significant $O_2^{\cdot-}$ scavenger. NQO1 may also stabilize p53, which is a transcription factor with tumor suppressor activity (Ross, 2004). The p53 protein has a major role in cessation of cell cycle and initiation of apoptosis, although a role for which in upregulation of glutathione peroxidase has also been reported (Tan et al., 1999). NQO1 and p53 share in numerous stimulants, and NQO1 has been shown to stabilize p53 and therefore promote apoptosis (Ross, 2004). Stabilization of p53 by NQO1 is likely one of the mechanisms of the anti-tumor and anti-carcinogenic properties of NQO1.

NQO1 has been found in high amounts in rat aortic smooth muscle cells, rat embryonic cardiac H9c2 cells, and human aortic smooth muscle and endothelial cells (Zhu et al., 2007a). In spite of the high levels of the enzyme in rat and mouse liver, human liver did not show appreciable amounts (Ross, 2004). In human tissues, NQO1 is primarily located in epithelial and endothelial cells. High levels of NQO1 in these tissues may provide

a mechanism by which NQO1 protects against harmful agents that are directed to the body from gut, airways, or vascular system. Nevertheless, upon over-exposure of cells to oxidative stress, considerable amounts of NQO1 can be expressed in other tissues.

2.8.2.1.2. Heme oxygenase-1

Heme oxygenases are rate-limiting enzymes that catalyze degradation of heme into biliverdin, carbon monoxide, and ferrous iron (Perrella & Yet, 2003; Chen & Kunsch, 2004; Katori et al., 2002). Three isoforms of heme oxygenase have been identified: HO-1, -2, and -3. HO-1 is a phase 2 protein and can be induced by a number of stimulators including heme, cytokines, nitric oxide donors, and ROS, as well as conditions of oxidative stress such as organ transplantation, atherosclerosis, and ischemia-reperfusion. Heme is a strong pro-oxidant and its degradation limits generation of ROS. High levels of heme can be released upon ischemia-reperfusion from damaged tissues and from injured red blood cells (Kumar & Bandyopadhyay, 2005; Katori et al., 2002; Tsuchihashi et al., 2004). The source of free heme in tissues is mainly heme-containing proteins such as myoglobin, catalase, superoxide dismutase, glutathione peroxidase, cytochromes, soluble guanylate cyclase, and nitric oxide synthases (Zhuang et al., 2003).

The first two mentioned products of heme breakdown by heme oxygenases, biliverdin and carbon monoxide, provide benefits for the cell. Biliverdin, which itself is an antioxidant, is rapidly reduced to bilirubin, a powerful peroxyl radical scavenger and one of the most important antioxidants in serum and tissues (Chen & Kunsch, 2004; Katori et al., 2002). Carbon monoxide is a vasodilatory, anti-inflammatory, and anti-apoptotic agent. The ferrous iron resultant from the activity of heme oxygenases could act as a co-factor for oxidative reactions. However, it is quickly picked up by endoplasmic reticulum and then exported from cells where it is captured by iron-binding proteins, such as ferritin, and quelled. Increased intracellular iron levels have been shown to up-regulate apoferritin protein synthesis. Indeed, the apoferritin up-regulation may be a part of the antioxidative response attributed to HO-1. Moreover, HO-1 up-regulates an ATP-dependent iron pump, which resides along with HO-1 in the microsomal membrane of the endoplasmic reticulum and mediates the transport of non-protein-bound iron from the cytoplasm into the microsomal vesicles, where it can be released to the vascular system by means of exocytosis.

Localization studies have revealed that the induction of HO-1 occurs in cardiac cells including those located around blood vessels (Sharma et al., 1999). The presence of HO-1 in pre-vascular areas suggests that carbon monoxide generated by HO-1 may play a role in regulating vascular tone. A considerable amount of HO-1 may also be expressed in monocytes/macrophages and myofibroblasts (Hangaishi et al., 2000).

HO-1 is believed to play a role in ischemia preconditioning of heart (Jancso et al., 2007; Sharma et al., 1999). Overexpressing HO-1 in myocardial cells has improved the outcome of ischemia-reperfusion resulting in better contractile recovery and improved hemodynamic parameters, smaller infarct area, less apoptosis and ventricular remodelling, reduced myocardial fibrosis and scarring, and suppressed inflammation (Perrella & Yet, 2003; Liu et al., 2006; Tang et al., 2005; Vulapalli et al., 2002; Yet et al., 2001; Hangaishi et al., 2000). Human HO-1 gene transfer to rats 8 weeks prior to coronary ligation reduced oxidative damage and infarct size, myocardial fibrosis and left ventricular remodeling, suggesting the method of HO-1 gene transfer as a potential treatment for patients at high risk of heart attack (Melo et al., 2002; Liu et al., 2006). HO-1 may also protect cardiac cells from oxidative injury caused by pressure stress (Perrella & Yet, 2003).

It has been shown that HO-1 remains unaffected in hearts during ischemia but is induced during reperfusion primarily by ROS, as ROS scavengers could inhibit its induction (Maulik et al., 1996). Hangaishi et al. (2000) detected an increase of HO-1 expression 24 h after the establishment of reperfusion, with a further elevation at 48 h, and a reduction thereafter. Furthermore, HO-1 expression increased, sometimes by 4 times, in non-fibrillating ischemic-reperfused hearts, but remained unchanged in fibrillating hearts, indicating a protecting effect of HO-1 against ischemia-induced ventricular fibrillation (Pataki et al., 2001; Bak et al., 2003).

2.8.2.1.3. γ -glutamylcysteine synthetase

The enzyme γ -glutamylcysteine synthetase, better known as glutamate cysteine ligase (GCL), catalyzes the rate-limiting step in GSH synthesis (Wu et al., 2004; Wild & Mulcahy, 2000; Dickinson & Forman, 2002). GSH is synthesized in two sequential ATP-dependent steps. In the first step, which is catalyzed by GCL, glutamate and cysteine are ligated through a peptidic bond between the γ -carboxyl group of the glutamate and the amino group of the cysteine. This γ -linkage protects GSH from degradation by intracellular proteases.

The only enzyme capable of breaking this γ -linkage is γ -glutamyl transpeptidase present at the outer surface of the plasma membrane and committed to transfer the glutamyl moiety of all forms of glutathione to other amino acids, thereby facilitating glutathione degradation. The second stage of GSH synthesis is catalyzed by glutathione synthetase which binds glycine to γ -glutamylcysteine and forms GSH.

The synthesis of GSH is primarily controlled by GCL (Wu et al., 2004; Wild & Mulcahy, 2000; Dickinson & Forman, 2002; Chen & Kunsch, 2004). Many factors such as oxidative and nitrosative stress, inflammatory cytokines, cancer and cancer therapy, and protein deficiency affect GCL expression or activity. For instance, oxidative stress has been identified as one of the stimulators of GCL activity. GSH itself exerts a negative feedback effect on GCL activity and GSH synthesis. The availability of cysteine has a positive impact on the synthesis. Glutamate, however, in high concentrations in plasma, as in some neurological diseases, may counteract GSH synthesis via competition with cysteine for entering cells (Wu et al., 2004). However, high intracellular glutamate concentrations, which occur rarely, enhance GSH synthesis.

GCL consists of two subunits, a heavy subunit with catalytic activity and a light subunit with regulatory activity (Soltaninassab et al., 2000; Wild & Mulcahy, 2000). The catalytic subunit is responsible for the enzymatic activity. It contains binding sites for the substrates, i.e. glutamate and cysteine, and is the site of feedback-inhibition by GSH. The regulatory subunit regulates the propensity of the catalytic subunit to bind substrates or to be inhibited by GSH. Interaction of the regulatory subunit with the catalytic subunit decreases the K_m for glutamate and increases the K_i for GSH inhibition. The numerous factors that can affect the activity of GCL exert their effect through activation or inhibition of either or both subunits. A mechanism by which the activation is achieved is through formation of disulfide bonds. It has been revealed that both catalytic and regulatory subunits of human GCL possess cysteine residues. Formation of at least 2 disulfide bonds in the catalytic subunit, one of them with the regulatory subunit, is necessary for the enzyme activity.

2.9. Flavonoids

Flavonoids are a subgroup of the more extended family of polyphenols. Polyphenols constitute a widespread group of plant compounds implicated in plants' wellbeing, growth and reproduction, pigmentation, and protection against micro-organisms and enemies (Bravo, 1998). They are widely distributed in plants from the root and stem to leaves, flowers, and fruits. Their quantities in fruit and vegetables are influenced by numerous factors including light, environmental conditions, plant species, degree of ripeness, germination, processing, and storage. As an example, cherry tomatoes possess six times more quercetin per gram fresh weight than normal size varieties of tomatoes, probably because polyphenols are generally synthesized and stored in the skin, and therefore smaller varieties have a higher skin to volume ratio (Crozier et al., 1997). Total dietary intake of polyphenols is firmly dependent on the food culture and the individual food preferences, but has been estimated to be about 1 g/day (Scalbert & Williamson, 2000) and that of flavonoids between 2 and 70 mg/day (Hertog et al., 1995). Moreover, it has been reported that 10% of the population and 30-70% of patients with specific diseases consume herbal medicines, which naturally contain considerable amounts of polyphenols (Galati & O'Brien, 2004).

There are more than 8000 polyphenolic compounds identified, each with a structure containing at least one phenol which is a hexagon ring (benzene) with a hydroxyl group (Svobodova et al., 2003). Based on their chemical structure, polyphenols can be divided into at least 10 different classes, one of the major groups of which is flavonoids (Bravo, 1998; Tapiero et al., 2002).

2.9.1. Classification

Flavonoids were discovered in the 1930's when a factor extracted from lemon juice could attenuate vessel permeability and bleeding in scorbutic guinea pigs where vitamin C was not effective, leading to their nomination as vitamin P although this terminology was later dismissed (Dragsted, 2003). More than 5000 flavonoids have been identified (Ross & Kasum, 2002). The basic structure of flavonoids consists of two benzene rings (A and B) with a pyran ring (C) in the middle (Figure 2.8-A). Flavonoids are divided into several subclasses including flavonols, flavanols (including proanthocyanidins), flavanones, flavones, isoflavones, and anthocyanins (Tapiero et al., 2002; Bravo, 1998).

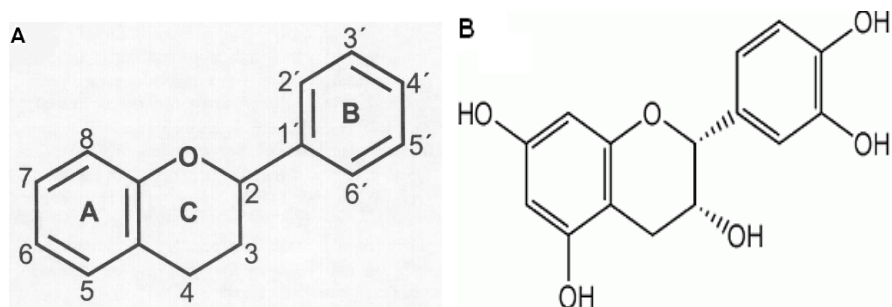


Figure 2.8. *A.* Basic structure of and numbering system of flavonoids. *B.* Epicatechin

Flavonols are represented by quercetin, kampferol, and myricetin (Tapiero et al., 2002; Hertog et al., 1997b). They are wide-spread in fruit and vegetables, and may contribute largely to our daily flavonoid consumption. Quercetin, one of the most studied flavonoids, is ingested especially through consumption of tea, onions, red wine, and apples.

Flavanols include catechins, which are largely found in green tea (Figure 2.8-B). Black tea has far less catechin than green tea due to oxidation of catechins during fermentation. Other major sources of flavanols are chocolate, apples, pears, grapes, and red wine (Gu et al., 2006). Proanthocyanidins or condensed tannins (Figure 2.9-A) are high-molecular weight oligomers and polymers of catechins, and are the most ubiquitous polyphenols in nature after lignans (Gu et al., 2004). The major sources of proanthocyanidins in the diet are chocolate, grapes, and apples.

Isoflavones, such as genistein and daidzein, are phytoestrogens and may be beneficial in prevention of breast and prostate cancer, menopausal symptoms, cognitive disabilities, osteoporosis, and heart diseases (Cooke, 2006). Soy bean is a rich source of isoflavones.

Anthocyanins (Figure 2.9-B), such as cyanidin, malvidine, and delphinidin, provide red and purple pigments for fruits. They are abundant in red and black cherries, berries, grapes, and legumes. Consumption of 200 g of black grapes and berries can afford about 1 to 1.5 g anthocyanins (Manach et al., 2005).

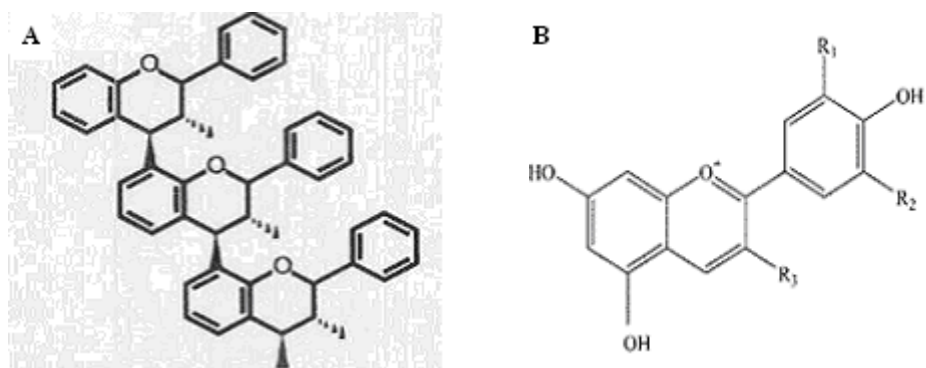


Figure 2.9. *A.* Proanthocyanidins. *B.* Anthocyanins.

2.9.2. Bioavailability and metabolism

In nature, flavonoids are generally present as glycosides, conjugated to sugars, although their aglycone forms may also exist (Ross & Kasum, 2002). The aglycones have stronger antioxidant activity than glycoside forms. The weakening of the antioxidant activity of flavonoids after glycosylation may be due to removing hydroxyl groups by conjugated glycosides, and thereby inhibiting them from scavenging ROS or chelating transition metals. Furthermore, as glycosylation enlarges the molecule, the passage through membranes may decrease upon glycosylation, leading ultimately to less antioxidant activity. Nonetheless, glycosylation enhances water solubility of the compound and subsequently improves its absorption from the gastrointestinal tract.

Flavonoid glycosides can be hydrolysed from sugar moieties by hydrolases at the intestinal brush border or by colonic micro-organisms (Tapiero et al., 2002; Depeint et al., 2002; Kroon et al., 2004). They may also be transported via a sodium-dependent glucose transporter into enterocytes where the sugar moieties are removed by β -glucosidases. After release from sugars, the flavonoid aglycones undergo conjugation reactions involving glucuronidation and sulfation with or without methylation. The conjugations occur in enterocytes and liver, in the latter as a part of detoxification processes. Conjugation facilitates their excretion and thereby shortens their plasma half-life. Almost all flavonoids

in plasma and urine are as conjugated forms. Thus, cells in the body are usually exposed to flavonoid metabolites and conjugates rather than aglycones.

The conjugates are physically and chemically different from aglycones, and thereby their biologic properties are also different (Kroon et al., 2004; Manach et al., 1998). For instance, as the conjugation blocks electron movement over the rings, the conjugated molecules display less potential for redox and therefore antioxidant activity, although this greatly depends on the position of the conjugation. One apparent example of the reduced antioxidant activity resulting from post-absorption structural modification of flavonoids is *O*-methylation of catechol group in the B ring which remarkably lowers the reactivity potential of the molecule for reaction with ROS (Pollard et al., 2006). Conjugation is, in fact, one of the defensive mechanisms of the body against flavonoids as pro-oxidants. As flavonoids possess antioxidant activity, they might also accompany oxidative consequences. By conjugation, the body reduces their antioxidant and potential pro-oxidant effects (Cano et al., 2002).

Flavonoids differ in the magnitude and velocity of absorption and the rate of elimination and plasma half-life (Manach et al., 2005). The information available on bioavailability and plasma kinetics of flavonoids is greatly variable. Overall, they have low intestinal bioavailability and rapid urinary and biliary excretion, and therefore with a regular diet their plasma concentration rarely exceeds 1 μ M (Scalbert & Williamson, 2000). Manach et al. (2005) according to the data provided by 97 bioavailability studies in humans suggested that the best rate of absorption among flavonoids is seen in gallic acid and isoflavones, followed by catechins, flavanones, and quercetin glycosides. Gallic acid although has a good absorption, its conjugation with catechins decreases the bioavailability of catechins.

Proanthocyanidins and anthocyanins have shown very low bioavailability (Manach et al., 2005). For instance, the absorption rate of anthocyanins from concentrated black current juice has been found less than 1% (Matsumoto et al., 2001). However, anthocyanin availability may have been underestimated because of technical complications in measuring anthocyanin metabolites (Manach et al., 2005). In contrast to other flavonoids that are separated from their glycosides during the absorption process, anthocyanins appear in plasma in unmodified glycosylated forms, although some glucuronide- and sulphate-conjugated forms in plasma have also been detected (Manach et al., 2005; Kroon et al., 2004, Dell'Agli et al., 2004). Polymerization markedly impairs the absorption of

proanthocyanidins (Manach et al., 2005; Spencer, 2003). Nevertheless, they may be cleaved to smaller units (i.e. monomers, dimers, etc) by gastric juice or by intestinal microflora, get absorbed, and yield some of the benefits that are currently attributed to proanthocyanidins. Urinary excretion of proanthocyanidin dimers has been reported in rats (Baba et al., 2002).

Quercetin metabolites are excreted very slowly, having a half-life of 11-28 h in plasma (Manach et al., 2005) probably due to tight binding to serum albumin (Dufour & Dangles, 2005). This may explain accumulation of quercetin in plasma during long-term supplementation (Manach et al., 2005). In this way, considerable concentrations of quercetin can be achieved through maintaining a regular diet with moderate amounts of quercetin. Contrarily, anthocyanins and catechins are excreted as rapidly as they are absorbed. For instance, anthocyanins reach the highest levels in plasma within 1-4 h and the maximum level in urine on average 2.5 h after ingestion. Although catechins have shown short plasma half-life and rapid elimination, they may still be capable of accumulating in plasma over a period of consumption (van der Hof et al., 1999), although they are still cleared as quickly as one day after stopping their consumption (Henning et al., 2006).

In vitro, the biological effects of flavonoids are achieved by concentrations (in the micromolar range) higher than those accessible in plasma *in vivo* (mostly in the nonomolar range) (Manach et al., 2005). However, flavonoid conjugates may bind to some cell receptors or cellular components, resulting in their accumulation after prolonged ingestion (Depeint et al., 2002). Moreover, as flavonoids are hydrophobic, they tend to partition into membranes, are accumulated and protected from rapid excretion (Brookes et al., 2002).

2.9.3. Beneficial effects of flavonoids on the cardiovascular system

2.9.3.1. Antioxidant capabilities

Generally speaking, flavonoids are strong antioxidants, and because of their antioxidant activity as well as their abundance in fruit and vegetables they may partly contribute to the currently known health benefits of plant foods. Studies have shown antioxidant effects of flavonoids in a wide range of oxidative systems. Their antioxidant activity appears to involve several mechanisms, one of which is electron/hydrogen donation facilitated by electron delocalization over the aromatic rings (Duthie & Crozier, 2000). The reaction rate constant for some flavonoids may even be more than that of vitamin E due to

the existence of multiple hydroxyl groups in the molecule and also because of the particular aromatic structure which supports extended delocalization of unpaired electrons. Figure 2.10 depicts a number of possible mechanisms by which flavonoids are postulated to protect heart against ischemia-reperfusion injury. We will talk about these effects effects in the next few sections.

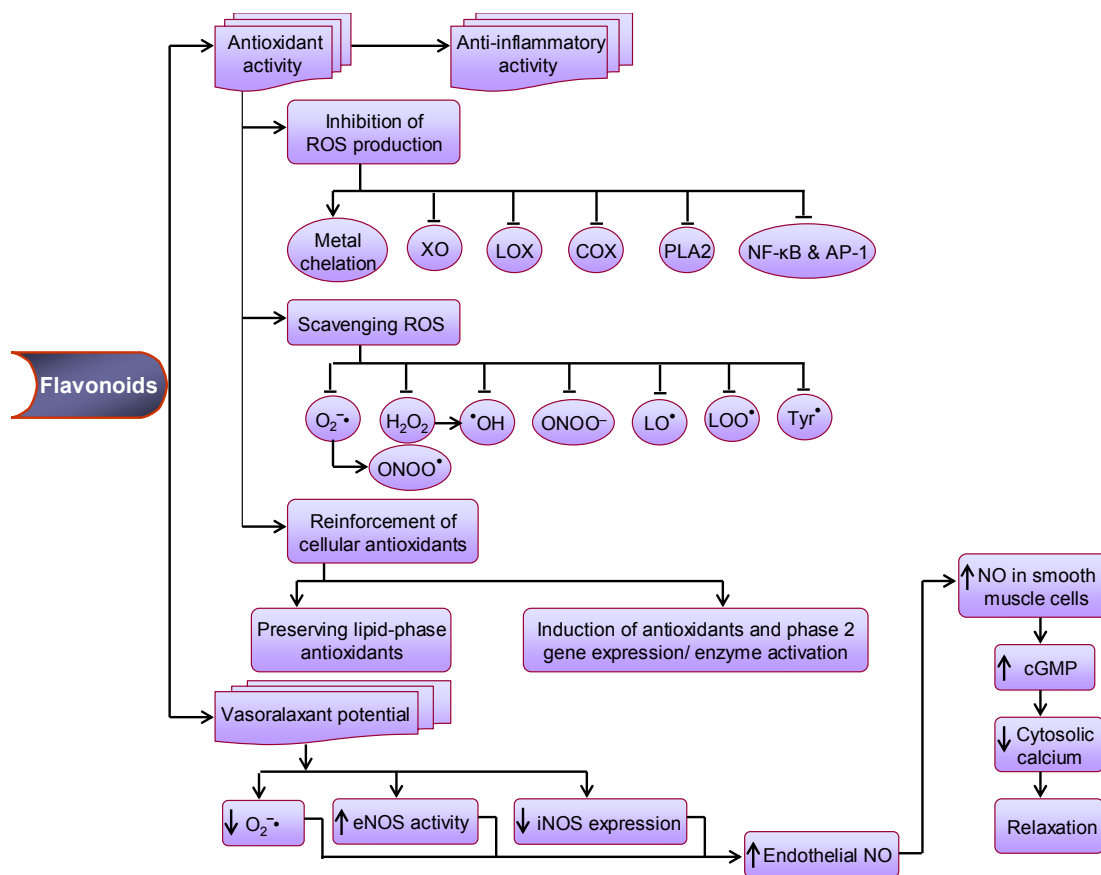


Figure 2.10. Biological properties of flavonoids that may help against myocardial ischemia-reperfusion injury.

Flavonoids may exert cardioprotective effects through various mechanisms including antioxidant activities, which inhibit inflammatory responses, and increase vasorelaxation capacities. For more information please refer to the Sections 2.9.3.1 to 2.9.3.3. **⊥** shows inhibition of enzyme activity or scavenging effect. XO, xanthine oxidase; LOX, lipoxygenase; COX, cyclooxygenase; PLA2, phospholipase A₂; Nf-κB, nuclear factor-kappa B; AP-1, activator protein-1; O₂^{•-}, superoxide radical; H₂O₂, hydrogen peroxide; •OH, hydroxyl radical; ONOO⁻, peroxynitrite; LO•, alkoxyl radical; LOO•, peroxy radical; Tyr•, tyrosyl radical; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; cGMP, cyclic guanosine monophosphate.

2.9.3.1.1. Superoxide radicals

Numerous studies have pointed to the ability of flavonoids, especially epigallocatechins, to scavenge superoxide radical ($O_2^{\cdot-}$) (i.e. Jovanovic & Simic, 2000; Cos et al., 1998; Chun et al., 2003; Maffei Facino et al., 1996). However, higher than *in vivo* attainable concentrations might be needed to enable flavonoids to play a considerable role in scavenging $O_2^{\cdot-}$, and it is more likely that other endogenous antioxidants provide a greater contribution in quenching intracellular $O_2^{\cdot-}$ (Huisman et al., 2004).

Flavonoids counteract $O_2^{\cdot-}$ by two modes of action: direct scavenging activity and inhibition of xanthine oxidase. Nonetheless, a specific molecular structure is seemingly required for manifestation of these and other activities. This implies that not all flavonoids possess antioxidant activity and that those which possess antioxidant activity do not necessarily follow the same mechanisms for exhibiting the antioxidant effect. By this way, it is very likely that a specific flavonoid inhibits xanthine oxidase activity but is incapable of scavenging $O_2^{\cdot-}$. More strikingly, it is possible that a particular structure exerts xanthine oxidase inhibition but at the same time stimulates $O_2^{\cdot-}$ production (Cos et al., 1998).

2.9.3.1.2. Peroxynitrite

A number of flavonoids including epicatechin, quercetin, myricetin, and proanthocyanidin oligomers have been shown to prevent peroxynitrite-induced oxidation and more effectively nitration reactions (Sadeghipour et al., 2005; Wippel et al., 2004; Arteel et al., 2000; Terao et al., 2001). In general, peroxynitrite ($ONOO^-$) has an unstable nature and can be protonated to peroxynitrous acid, which is quickly cleaved to toxic reactive chemicals, such as nitrogen dioxide and hydroxyl radical, which can nitrate and oxidize a variety of cellular molecules including proteins and DNA. Alternatively and predominantly, $ONOO^-$ can react with carbon dioxide, yielding ultimately nitrogen dioxide and carbonate radicals that both have very high rate constants for reaction with biomolecules and can initiate many oxidation and nitration reactions (McCarty, 2007; Pacher et al., 2007). A nitration target for $ONOO^-$ is tetrahydrobiopterin, one of the NOS cofactors, resulting in impaired NOS activity and endothelium dysfunction. $ONOO^-$ -induced inhibition of prostacyclin synthetase also produces endothelial dysfunction. Accordingly, scavenging $ONOO^-$ by flavonoids is particularly useful for maintaining endothelial function.

The mechanism of flavonoid protection against ONOO^- is not yet fully understood. Some of the protective effects of flavonoids against ONOO^- may result from their action on $\text{O}_2^{\cdot-}$, one of the precursors of ONOO^- formation. As it was mentioned, flavonoids can inhibit $\text{O}_2^{\cdot-}$ by either scavenging $\text{O}_2^{\cdot-}$ or inhibiting xanthine oxidase activity. Moreover, flavonoids may, through suppression of inflammatory reactions, inhibit activation of neutrophils, which upon activation generate considerable amounts of $\text{O}_2^{\cdot-}$ and nitric oxide (Paulino et al., 2006). Catechol-containing flavonoids, such as epicatechin and quercetin, can inhibit ONOO^- -induced oxidation reactions, being themselves transformed into quinone and *o*-quinone, which can then be conjugated to glutathione, yielding glutathionyl adducts, and excreted (Pollard et al., 2006). On the other hand, reaction of ONOO^- with methylated and conjugated flavonoids results in formation of mono- and di-nitrated and nitrosated products (nitration products). The mechanism of flavonoid inhibition of nitration products is a matter of debate; whereas some have suggested a direct effect for flavonoids through competition with tyrosine to be nitrated by ONOO^- (Pannala et al., 1997; Kerry & Rice-Evans, 1999), others pose the possibility of flavonoid interaction with ONOO^- reaction intermediates such as tyrosyl radicals (Klotz & Sies, 2003). Yokozawa et al. (2003) reported that oral administration of epicatechin gallate to rats for 3 weeks decreased plasma concentration of nitrotyrosine which had been elevated as a result of lipopolysaccharide administration plus renal ischemia-reperfusion. Little is known about the biological effects of nitration products of flavonoids although some have pointed to their mutagenic as well as chemotherapeutic properties (Pollard et al., 2006).

Flavonoids may be able to scavenge ONOO^- at concentrations that are achievable *in vivo* (McCarty, 2007). Cyanidin-3-*O*-glucoside at concentrations as low as 8.5 nM has been shown capable of ameliorating ONOO^- damaging effects on endothelium, such as DNA damage and defective relaxation (Serraino et al., 2003).

2.9.3.1.3. Peroxyl radicals and lipid peroxidation

Flavonoids can inhibit lipid peroxidation through either electron donation and blocking lipid oxidation chain reactions (Ozgova et al., 2003) or inhibiting lipoxygenases, which catalyze enzymatic lipid peroxidation in cellular membranes (Cos et al., 2004). Flavonoids are known as scavengers of peroxyl radicals (Nakao et al., 1998; Sato et al., 1999) and have shown ability to protect low density lipoprotein (LDL) and plasma lipids

from oxidation (Day et al., 1997; Boadi et al., 2005), in some reports being even more powerful than trolox (Plumb et al., 1998). Six weeks consumption of flavonoids led to attenuation of atherosclerotic lesions in apolipoprotein E deficient mice, lowered *ex vivo* LDL uptake by macrophages, and reduced *ex vivo* LDL susceptibility to oxidation despite unmodified levels of plasma cholesterol (Hayek et al., 1997). Similarly, flavonoids from pomegranate juice counteracted development of atherosclerotic lesions in atherosclerotic mice and inhibited macrophage-induced oxidation of LDL (Aviram et al., 2002).

However, the feasibility of consuming effective dietary doses of flavonoids must also be taken into consideration as the concentrations that are usually used in *in vitro* or animal studies are quite high and achieving the same doses by dietary intake is hard or impossible. For instance, green tea polyphenols have been able to reduce blood cholesterol levels in hypercholesterolemic and blood pressure in hypertensive animals, but at doses achievable by 20 cups of green tea per day for humans (Hara, 1992). Similarly, Lotito & Frei (2004) reported that despite the fact that apple extract prolonged the half-life of plasma antioxidants (urate and α -tocopherol but not ascorbic acid) and delayed lipid peroxidation *in vitro*, consumption of apples (5 in one dose) by humans did not produce the same benefits. Also, one-time ingestion of proanthocyanidin-rich chocolate by individuals, although it increased the concentration of epicatechin in plasma, did not cause a significant change in plasma antioxidant capacity or plasma lipid peroxidation indices (Wang et al., 2000). Likewise, controlled clinical trials have failed to demonstrate benefits of drinking 5-6 cups of tea per day for 1-4 weeks on cholesterol levels and blood pressure (Bingham et al., 1997; Hodgson et al., 1999), although more prolonged intakes of tea as a dietary habit may deliver some benefits (Stensvold et al., 1992).

2.9.3.1.4. Hydroxyl radicals and hydrogen peroxide

Studies have also indicated the inhibitory effect of flavonoids such as myricetin, quercetin, morin, and proanthocyanidins on hydroxyl ($\cdot\text{OH}$) radicals (Chen et al., 2002; Zou et al., 2002; Jung et al., 2003; Maffei Facino et al., 1996; Sato et al., 1999). *In vivo*, a part of the inhibitory effect of flavonoids on $\cdot\text{OH}$ is probably exerted through blocking generation of $\cdot\text{OH}$ in the Fenton reaction by means of scavenging $\text{O}_2^{\cdot-}$ and H_2O_2 and/or chelating transition metals. A protective effect of flavonoids against hydrogen peroxide-induced

cytotoxicity has also been reported (Melidou et al., 2005; Dajas et al., 2003; Estany et al., 2007).

2.9.3.1.5. Metal chelation

One of the mechanisms by which flavonoids confer their antioxidant effect is chelation of metal ions, such as iron and copper (Mandel et al., 2006; Gabrielska & Oszmianski, 2005; Morel et al., 1993; Maffei Facino et al., 1996). This property of flavonoids is in agreement with the well-known phenomenon of binding metals by flavonoids and polyphenols in the gut which negatively affects the bioavailability of these metals and may lead to mineral deficiencies (Bravo, 1998). Transition metal ions are critical co-factors of the Fenton reaction, which generates $\cdot\text{OH}$ from H_2O_2 , and therefore their chelation by flavonoids makes them unavailable for this kind of reaction. Melidou et al. (2005) found that flavonoid protection of cells against H_2O_2 -induced DNA damage was in good correlation with the iron binding capacity of flavonoids rather than the electron donating potential. Interestingly, it has been suggested that specific flavonoids upon binding metals may behave as a superoxide dismutase, scavenging $\text{O}_2^{\cdot-}$, more potent than the parent flavonoids while devoid of catalytic activity for conversion of H_2O_2 to $\cdot\text{OH}$ (Kostyuk et al., 2007; Malesev & Kuntic, 2007). Flavonoids can bind metals in metal:flavonoid ratios of 1:1, 1:2, 2:2, and 2:3 (Fernandez et al., 2002).

2.9.3.1.6. Interaction of flavonoids with other cellular antioxidants

It was pointed out in the previous sections that lipophilic antioxidants are likely to be the last antioxidants that are depleted upon exposure to oxidative stress, and that other cellular antioxidants such as ascorbic acid and glutathione are at the front line of defence against oxidants. It has been suggested that flavonoids can act as intermediate antioxidants, protecting lipophilic antioxidants and being protected by hydrophilic antioxidants (Lotito & Fraga, 2000 & 1999). For instance, catechins did not prevent ascorbic acid oxidation in human plasma treated with a peroxy radical generator, but they reduced oxidation of α -tocopherol and β -carotene, and lessened the rate of lipid peroxidation. Catechin oxidation did not occur unless the existing ascorbic acid was totally oxidized. In the presence of metal ions, however, flavonoids can prevent oxidation of ascorbic acid through a chelating

mechanism (Clemetson & Andersen, 1966), although this may not be physiologically relevant but may be of benefit in pathological circumstances.

Despite compelling evidence indicating the occurrence of ascorbic acid depletion prior to flavonoid oxidation under situations of oxidative stress, in non-pathological conditions flavonoids can elevate concentrations of ascorbic acid as is shown by proanthocyanidins which increased plasma levels of ascorbic acid and reduced those of vitamin E (Maffei Facino et al., 1999). The decrease of the vitamin E plasma level in this case may be an outcome of an inhibitory effect of ascorbic acid on the release of vitamin E from the liver.

2.9.3.1.7. Flavonoid induction of phase 2 enzymes

A large number of studies have focused on the effect of flavonoids, mostly stimulatory, on phase 2 enzymes. Because detoxifying xenobiotics and carcinogens is the major commitment of phase 2 enzymes, induction of phase 2 enzymes by flavonoids has been posed as one of the mechanisms of the chemopreventive effects of flavonoid application (reviewed in Moon et al., 2006). Flavonoid induction of phase 2 enzymes is flavonoid-, enzyme-, and cell/organ type-specific. This means that inducing a particular phase 2 enzyme by a certain flavonoid in a specific organ does not mean that the enzyme could be induced by other flavonoids in the same organ, or by the same flavonoid in other organs, or that the same flavonoid could induce other phase 2 enzymes in the same cell and organ type. Interestingly, an inhibitory effect of quercetin on glutathione S-transferase P1-1 has also been indicated, probably as a result of inhibitory effect of quercetin oxidation products with critical cysteine residues of the enzyme (van Zanden et al., 2003).

2.9.3.1.7.1. NAD(P)H:quinone oxidoreductase

Increased activity and expression of NAD(P)H:quinone oxidoreductase (quinone reductase) has been seen with application of a number of flavonoids in a variety of cells. For instance, one month administration of green tea in drinking water (0.2%, w/v) increased quinone reductase activity in small bowel, liver, and lungs of SKH-1 hairless mice (Khan et al., 1992). Similarly, buccal pouch and liver quinone reductase activities were increased over 14 weeks administration of 0.05% green and black tea polyphenols in the diet (Chandra Mohan et al., 2005). Epigallocatechin gallate and epicatechin gallate have shown a powerful

induction of antioxidant response element (ARE)-mediated gene expression in human cervical carcinoma HeLa cells (Chen et al., 2000a). The induction of the ARE appeared to be structurally related to the gallate group at position 3. Similarly, dietary administration of ellagic acid to rats caused a 9-fold increase in hepatic and 2-fold increase in pulmonary quinone reductase activity, and an 8-fold increase in hepatic quinone reductase mRNA level (Barch & Rundhaugen, 1994). Chlorogenic acid also increased enzyme activities of glutathione S-transferase and quinone reductase in the JB6 mouse epidermal cell line (Feng et al., 2005). In contrast, two weeks gavage administration of a number of flavonoids including quercetin (100 mg/kg) to rats did not considerably affect the activity of quinone reductase in heart, liver, and colon, except for tangeretin and chrysin which caused a 2-fold increase in heart and colon, respectively (Breinholt et al., 1999).

Induction of phase 2 enzymes by flavonoids has not always yielded desirable outcomes. For instance, treatment of a rat hepatocyte cell line with anthocyanins especially cyanidin, delphinidin, malvidin, and kuromanin elevated activities of quinone reductase and a number of glutathione-related enzymes, but it was not remarkably associated with prevention of H₂O₂-induced cell death (Shih et al., 2007). Also, incubation with higher concentrations of anthocyanins decreased activities of phase 2 enzymes concomitantly with activation of death signals (Srivastava et al., 2007).

2.9.3.1.7.2. Glutamate cysteine ligase

Little data are available regarding flavonoid impact on glutamate cysteine ligase (GCL). Myhrstad et al. (2002) reported induction of the catalytic subunit of GCL by onion extracts, quercetin, kampferol, or apigenin, but not myricetin although it has a very similar structure to quercetin, in COS-1 cells (monkey kidney cell line). Also, feeding mice with berries for 3-4 weeks elevated induction of GCL in skeletal muscle and brain but decreased it in liver, although these alterations were only seen in half of the mice (called responders) and GCL in the other half was not changed by berries (Carlsen et al., 2003). However, in spite of the induction of GCL in some organs by berries, the concentration of glutathione only increased in gastrocnemius muscle (Moskaug et al., 2005). The reason that merely half of the animals exhibited induction of GCL in response to berries could be the variability of intestinal absorption and the effect that gut micro-organisms can exert on physical and biological characteristics of flavonoids.

2.9.3.1.7.3. Heme oxygenase-1

Similar to GCL, none of the studies available in the literature has examined the promotive effect of flavonoids on heme oxygenase (HO)-1 in heart cells. Here are reports on other cell types. Szabo et al. (2004) treated rats orally with flavonoids from sour cherry seed for 2 weeks, and then subjected their retina to ischemia-reperfusion. HO-1 expression and activity was reduced as a result of ischemia-reperfusion, and 10-30 mg/kg of flavonoid-rich extract of sour cherry could prevent these reductions. In cell culture studies, flavonoids (fisetin, myricetin, quercetin, eriodictyol, toxifolin, epicatechin, and epigallocatechin gallate) induced expression of both Nrf2 and HO-1 in human retinal pigment epithelial cells (Hanneken et al., 2006). Also, incubation of bovine aortic endothelial cells with chalcone induced expression of HO-1 and increased nuclear accumulation of Nrf2 (Liu et al., 2007b). Similarly, quercetin induced expression of HO-1 in BV-2 microglia cells (Chen et al., 2005). Hydroxychalcone also showed an ability to induce expression of HO-1 in RAW 264.7 macrophages (Abuarqoub et al., 2006).

2.9.3.2. Vasorelaxation

Besides antioxidant effects, flavonoids possess other properties that help in alleviation of ischemia-reperfusion injury, for instance, through better re-establishment of blood flow in post-ischemic hearts. The vasorelaxation effect has mostly been investigated for the family of polyphenols rather than the flavonoid subclass. A variety of polyphenols have shown a capacity to induce relaxation in blood vessels. Their mechanisms of action are varied and may be exerted in endothelium-dependent or -independent fashions. It has been shown that some polyphenols, such as quercetin and resveratrol, could induce vasorelaxation by both mechanisms (Chen & Pace-Asciak, 1996), although in the absence of endothelium much higher concentrations of polyphenols are required (Andriambeloson et al., 1997). The endothelium-dependent relaxation is mediated by NO. NO, which is produced in the endothelium, is delivered to smooth muscle cells where it manifests its biological function (Martin et al., 2002). An up-regulatory effect of polyphenols on NO levels has been indicated through activation of eNOS or by removing $O_2^{\cdot-}$, thereby inhibiting consumption of NO in the reaction to form $ONOO^-$ (Huk et al., 1998; Benito et al., 2002; Pechanova et al., 2004). As eNOS is a calcium-dependent enzyme, elevation of intracellular Ca^{2+} has been

suggested as the mechanism of the endothelium-dependent NO-mediated vasorelaxation by polyphenols (Stoclet et al., 1999; Zenebe et al., 2003; Martin et al., 2002; Andriambeloson et al., 1999). The Ca^{2+} rise, which was mediated by increased production of $\text{O}_2^{\cdot-}$ by polyphenols and inhibited by application of superoxide dismutase plus catalase (Duarte et al., 2004), was due to stimulating Ca^{2+} entry from extracellular milieu and Ca^{2+} release from intracellular stores (Martin et al., 2002).

Polyphenol-stimulated NO production activates guanylate cyclase which synthesizes cyclic GMP (cGMP) (Dell'Agli et al., 2004; Fitzpatrick et al., 1993). cGMP, which is an important mediator of vasodilation, acts by activating protein kinases which phosphorylate and activate a number of target proteins including those involved in ion channels. Activating these channels leads to Ca^{2+} removal from the cytosol to either endoplasmic reticulum or the extracellular environment (Nauli et al., 2001). The resulting low intracellular Ca^{2+} mitigates cellular contractility and produces relaxation. In contrast to the aforementioned mechanism for polyphenol-induced vasorelaxation, inhibition of NO-cGMP-mediated vasorelaxation has also been observed by some flavonoids (Huang et al., 2004).

The mechanism of endothelium-independent relaxation by polyphenols is as yet uncertain. However, it is likely that signaling pathways downstream from cGMP are activated in smooth muscle cells independently of NO. Among downstream mechanisms are regulating activity of protein kinases and phosphodiesterases (a family of enzymes responsible for the breakdown of the vasorelaxants cyclic AMP (cAMP) and cGMP), inhibition of the Ca^{2+} influx from extracellular and intracellular resources, and activation of voltage-dependent potassium channels (Chan et al., 2000; Ajay et al., 2003; Novakovic et al., 2006). The blockade of extracellular Ca^{2+} influx and Ca^{2+} release from endoplasmic reticulum may be one of the possible mechanisms of flavonoid protection in states of ischemia-reperfusion.

Other mechanisms of flavonoid relaxation of blood vessels are stimulating production of prostacylin PGI_2 (Aldini et al., 2003), increased activity, but not expression, of eNOS (Benito et al., 2002), inhibiting expression of iNOS but increasing expression and/or activity of eNOS (Hung et al., 2004; Olszanecki et al., 2002), augmenting NO half-life by scavenging $\text{O}_2^{\cdot-}$ (Huk et al., 1998), inhibition of the renin-angiotensin system (Cos et al., 2004), and scavenging peroxynitrite and therefore preserving tetrahydrobiopterin, the cofactor of NOS (McCarty, 2007).

Studies in both humans and animals have shown that supplementing the diet with flavonoids can result in vasodilation. However, it is not yet clear whether this can be achieved through a high-flavonoid diet as concentrations that have caused relaxation are much higher than plasma levels of flavonoids attainable by diet (McCarty, 2007).

2.9.3.3. Anti-inflammatory and anti-aggregatory effects

Similar to other properties of flavonoids, anti-inflammatory effects of flavonoids depend on the type of flavonoid and are extremely different from one flavonoid to another (Kim et al., 2004). Flavonoids have shown the capacity to suppress the enzymes involved in eicosanoid pathways, such as phospholipase A₂, cyclooxygenases, and lipoxygenases, thereby inhibiting production of inflammatory mediators. Further, flavonoids may inhibit production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and interferon- γ , and chemotactic agents. Flavonoids may also have an inhibitory effect on expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (Kris-Etherton et al., 2004; Gonzalez-Gallego et al., 2007). Many of these effects are exerted through blocking activities of enzymes implicated in signaling pathways especially protein kinase C and mitogen-activated protein kinase (MAPK), with downstream inhibition of transcription factors NF- κ B and AP-1 (Manthey, 2000; Gonzalez-Gallego et al., 2007), although stimulation of NF- κ B-induced gene expression by some flavonoids has also been reported (Cos et al., 2004). Flavonoid inhibition of protein kinase C has been suggested to occur through competitive binding of flavonoids with ATP at the active site of the enzymes (Manthey, 2000). Elevation of cAMP secondary to inhibition of phosphodiesterases has also been suggested as the mechanism of the anti-inflammatory effects of flavonoids (Manthey, 2000). Inhibition of phosphodiesterases probably occurs due to structural similarity of the purine ring of cAMP and benzopyran structure of flavonoids (Ferrell et al., 1979).

Schramm et al. (2001) reported that one-time consumption of high-proanthocyanidin chocolate decreased the plasma leukotriene to prostacyclin ratio concomitantly with an increase in plasma epicatechin. The leukotriene to prostacyclin ratio was also decreased in human aortic endothelial cells after incubation with proanthocyanidins. The reduction of leukotrienes likely results from inhibition of lipoxygenases (Selmi et al., 2006; Sies et al., 2005). Lipoxygenases possess an active ferric form of iron which is required for their

catalytic activity (Schewe et al., 2001). The activity of the lipoxygenases is abolished if the ferric iron is reduced to the ferrous form. ROS can activate the enzyme, while flavonoids have been suggested to inactivate it through either scavenging ROS or reducing the active ferric form of the enzyme.

Administration of flavonoids can mitigate vascular permeability via reduction of the number of leukocytes adherent to the endothelium (Bouskela & Donyo, 1997). Epigallocatechin gallate at physiologic concentrations reduced neutrophil migration into cultured human endothelial cells (Riemersma et al., 2001). Similarly, proanthocyanidins decreased expression of VCAM-1, but not ICAM-1, and mitigated leukocyte-endothelial cell interaction (Sen & Bagchi, 2001). Similar results have been observed in human studies. Taking 100 mg/day proanthocyanidins for one month decreased plasma concentrations of ICAM-1, VCAM-1, and E-selectin in systemic sclerosis patients (Kalfin et al., 2002). Moreover, cocoa flavanols inhibited IL-2 expression in stimulated leukocytes (Selmi et al., 2006). However, the response of inflammatory substances to flavonoids may be diverse, as for example small molecule proanthocyanidins (dimers, trimers) suppressed, while comparably larger molecules of proanthocyanidins stimulated, expression of IL-1 β . Contrary to this, small molecular weight proanthocyanidins were incapable of exhibiting endothelium-dependent relaxation in rabbit aortic rings, whereas large polymers demonstrated this kind of vasodilation.

Flavonoids have also been shown to inhibit platelet activation and aggregation (Cos et al., 2004; Manthey, 2000). As an example, intake of proanthocyanidin- and epicatechin-rich cocoa decreased markers of platelet activation and function, leading to inhibition of platelet aggregation and prolongation of blood coagulation time (Rein et al., 2000). The anti-platelet effect of flavonoids may be due to increased production of prostacyclin which via synthesis of cAMP reduces platelet aggregation (Kris-Etherton & Keen, 2002). Flavonoids may also decrease platelet activation through inhibition of phosphodiesterases responsible for degradation of cAMP (Manthey, 2000). Furthermore, given that NO has a protective role in maintaining a non-adhesive endothelium (Bertuglia & Giusti, 2003), and considering that flavonoids are stimulators of NO generation, they may inhibit adhesion of leukocytes and platelets to the endothelium through up-regulation of NO. Moreover, as inflammatory responses are greatly induced by oxidative stress, flavonoid inhibition of inflammation may be at least partly due to attenuation of oxidative stress.

2.9.4. Epidemiological studies

Epidemiological studies on the association of flavonoid consumption and the risk of cardiovascular disease are subject to controversies. Although in most cases an elevated risk of cardiovascular diseases has been associated with low intakes of flavonoids, there are also some reports rejecting this hypothesis or even documenting the opposite to it. Here are examples of the inconsistencies.

A 5- and 10-year follow-up study of men aged 65-84 years in Zutphen Netherlands between 1985 and 1995 revealed that flavonoid intake, mainly from black tea, onions, and apples, was inversely linked with mortality from coronary heart disease and less strongly with incidence of myocardial infarction and all-cause mortality (Hertog et al., 1993 and 1997a). The results also showed a dose-response negative correlation between flavonol intake and the risk of death from cardiovascular diseases and a first heart attack. Similarly, a 25-year follow-up of 16 cohorts from the Seven Countries Study demonstrated that the average consumption of flavonoids was inversely associated with death caused by heart disease (Hertog et al., 1995). More interestingly, the analysis of data revealed that flavonoid intake alone explained nearly 25%, and flavonoid intake along with the incidence of smoking explained 90% of the differences in the rates of coronary heart disease among the 16 cohorts. Also, moderate tea consumption has been found associated with lower prevalence of ventricular arrhythmias in patients with acute myocardial infarction (Mukamal et al., 2006). There are further studies documenting an inverse relationship between the amount of daily flavonoid intake and the incidence and mortality from coronary heart disease (Mojzisova & Kuchta, 2001).

However, there are also epidemiological studies that contest the benefits of flavonoids in human populations. For instance, a 14-year follow-up study on 1900 Welsh men aged 45-59 years revealed no association between flavonol consumption (mainly received from black tea) and the incidence of ischemic heart disease. Contrariwise, flavonol intake had a mild positive correlation with mortality from infarction and cancer and a strong relation with total cause mortality (Hertog et al., 1997b). The authors assumed that milk that is a customarily added to tea in the United Kingdom may have limited flavonoid absorption. Nevertheless, addition of milk to black tea (15 ml milk to 135 ml tea) has not been shown to change bioavailability of tea flavonols (Hollman et al., 2001). Consistent with Hertog and colleagues (1997b), a 5-year study on 34,789 male professionals aged 40-75 years showed

no association between flavonoid intake and incidence of nonfatal myocardial infarction, but raised the possibility that flavonoids reduce risk of mortality in people with a medical history of coronary heart disease (Rimm et al., 1996).

2.9.5. Flavonoid cytotoxicity

While describing beneficial features of flavonoids, their possible damaging effects should not be ignored. In fact, any kind of antioxidant could potentially act as pro-oxidant, if after delivering its antioxidant effect its oxidized form is not scavenged properly. The pro-oxidant effect of flavonoids become especially manifest in the presence of transition metals (Galati & O'Brien, 2004). Generally speaking, flavonoids upon exposure to various forms of electrophiles and ROS donate one or more electrons with concomitant oxidation to phenoxyl radicals, semiquinones, and quinones. These oxidized forms of flavonoids must be scavenged by ascorbate in order to be converted back to their original forms (Jovanovic & Simic, 2000) or conjugated to glutathione and excreted (Galati et al., 2001); otherwise they will initiate oxidative reactions (Galati & O'Brien, 2004).

In the presence of transition metal ions, the redox reactions including those of flavonoids are accelerated, generating greater amounts of radicals and reactive species. For instance, in the presence of flavonoids, the generation of hydroxyl radicals by metal-catalyzed Fenton reactions increases (El Hajji et al., 2006). It has been found that addition of copper ions accelerates auto-oxidation of quercetin, producing large amounts of H_2O_2 , while addition of iron attenuates quercetin auto-oxidation as iron-quercetin complexes seem to be relatively more stable.

Surprisingly, cytotoxic effects of flavonoids may not originate from stimulating formation of reactive oxygen species as some studies failed to prevent cytotoxic and DNA-damaging effect of flavonoids by antioxidants (Sasaki et al., 2007; Watjen et al., 2005). For instance, Sasaki et al. (2007) reported that quercetin at moderate concentrations (33 μM) induced death in PC12 neuronal cell line, and that N-acetylcysteine and glutathione could not inhibit its toxicity, although N-acetylcysteine and glutathione prevented H_2O_2 -induced cell death. Interestingly, quercetin did not protect cells against H_2O_2 cytotoxicity but rather exacerbated it.

Other possible explanations for flavonoid cytotoxicity may be perturbation in cellular normal metabolism including inhibition of enzymes such as protein kinase C and DNA topoisomerases that leads to cell demise (Middleton et al., 2000).

Some authors have suggested that cytoprotective concentrations of flavonoids are generally lower than their cytotoxic concentrations, for some flavonoids such as quercetin as much as 5-10 times (Watjen et al., 2005). However, depending on the circumstances of oxidative stress, the opposite may occur. For instance, Yamanaka et al. (1997) found that 1.5 μ M of catechins inhibited LDL oxidation when added in the initiation phase of LDL oxidation, but hastened it if added later in the propagation phase, when higher concentrations of catechins were effective in lowering oxidation.

The cytotoxic potential of flavonoids is complicated and it is hard to predict whether a particular flavonoid under specific circumstances exhibits anti- or pro-oxidant effect. Even under similar conditions and with similar concentrations, some flavonoids may exhibit antioxidant behaviour while others act as pro-oxidant (Choi et al., 2003). Some flavonoids have exhibited antioxidant activity against peroxynitrite-induced DNA strand cleavage, but they mostly also were capable of causing DNA strand breaks when applied in combination with NO (Ohshima et al., 1998). Environmental conditions are also intervening. For example, slightly alkaline pH, such as that in the intestine, has been shown to enhance auto-oxidation of flavonoids (Duarte Silva et al., 2000).

2.9.6. Flavonoid protection of heart against ischemia-reperfusion injury

Because the number of studies examining the effect of flavonoids on myocardial ischemia-reperfusion injury is large, here are presented only those which are relevant to our study.

2.9.6.1. Catechins

Catechins and the most abundant source of catechins, i.e. teas, have attracted much attention in the field of ischemia-reperfusion. Townsend et al. (2004) found that oral administration of 0.1% green tea extract in drinking water for 7 days or perfusing isolated hearts with epigallocatechin gallate for 30 min before global ischemia (35 min) and reperfusion (2 h) reduced myocardial apoptosis and infarct size. Green tea extract and

epigallocatechin gallate also improved hemodynamic markers including left ventricular systolic and diastolic pressure and reduced expression of Fas, active caspase-3, and phosphorylated p38 proteins. Potenza et al. (2007) fed SHR hypertensive rats for 3 weeks with food supplemented with 200 mg/kg/day epigallocatechin gallate, and then exposed the isolated hearts to 30 min ischemia and 2 h reperfusion. Hearts from treated animals had better coronary flow, limited infarct size, and improved post-ischemic ventricular recovery determined by higher left ventricular developed pressure and lower left ventricular end diastolic pressure. At least a part of the beneficial effects of epigallocatechin gallate has been postulated to be NO-dependent (Potenza et al. 2007; Hotta et al., 2006).

Similarly, Modun et al. (2003) treated rats orally with 250 mg/kg/day catechin for 10 days before isolation of hearts and induction of ischemia (30 min) and reperfusion (30 min). Catechin decreased the incidence and duration of ventricular fibrillation, improved left ventricular developed pressure while reducing left ventricular diastolic pressure, ameliorated cardiac performance, and lowered tissue lipid peroxides and lactate dehydrogenase released into the effluents. Catechins have also shown to protect the heart against chronic ischemic injury. Suzuki et al. (2007) administered 20 mg/kg/day tea catechins to rats orally during 28-day chronic ischemia, and found improved cardiac function and blood pressure, and decreased infarct size and thickening of the left ventricular inner wall in the tea-fed group. Catechins also reduced the number of infiltrating neutrophils into the myocardium, lowered expression of adhesion molecules and chemoattractants, and inhibited activation of NF- κ B and AP-1 as well as matrix metalloproteinase.

Perfusing isolated hearts with catechins has also shown benefits. Hirai et al. (2007) perfused hearts with epigallocatechin gallate and gallocatechin gallate for 4 min before ischemia (40 min) and throughout reperfusion (40 min). Catechin-treated hearts had lower abnormalities of blood pressure, preserved ATP levels, and less cytosolic Ca^{2+} concentrations. Catechins also prevented activation of caspase-3 in a different preparation of ischemic-reperfused hearts and inhibited Ca^{2+} uptake by isolated mitochondria incubated in a high Ca^{2+} medium. Addition of catechin to perfusate also inhibited elevation of low molecular weight iron and preserved ascorbic acid in hearts subjected to 10 min global ischemia and 5 min reperfusion (van Jaarsveld et al., 1996). Intravenous application of catechins has also been advantageous. Aneja et al. (2004) reported that intravenous administration of epigallocatechin gallate at the end of coronary artery occlusion (30 min) and during reperfusion (2 h) resulted in milder histological damage and necrosis, lower

plasma concentrations of creatine phosphokinase and interleukin-6, less incidence of neutrophil infiltration, and reduced activation of NF- κ B and AP-1 in rats.

2.9.6.2. Proanthocyanidins

Proanthocyanidins whose basic structure is similar to catechins have also shown benefits. Pataki et al. (2002) administered 50 and 100 mg/kg/day oligomeric proanthocyanidins from red grape seeds for 3 weeks to rats, and then subjected the hearts to 30 min global ischemia and 2 h reperfusion. Hearts in the treated groups had a dose-dependent lower incidence of ventricular tachycardia and fibrillation, greater left ventricular developed pressure, and better recovery of coronary and aortic flow, but no improvement in the heart rate. Proanthocyanidin-treated animals also had lower hydroxyl radicals released to the heart effluents. The number of apoptotic cells was fewer, the infarct area was smaller, and the amount of malondialdehyde released into the effluents at the beginning of reperfusion was lower in proanthocyanidin group (Sato et al., 1999 & 2001). Similarly, supplementation of the diet with 1% oligomeric proanthocyanidins for 3 weeks reduced the duration of ventricular fibrillation and lowered lactate dehydrogenase release into coronary effluents of hearts exposed to 20 min global ischemia and 30 min reperfusion (Al Makdessi et al., 2006).

Adding proanthocyanidins to the perfusate has also shown promising results. In a model of low-flow (1 ml/min) ischemia (40 min), perfusing hearts with oligomeric proanthocyanidins improved cardiac contractile function, decreased arrhythmias, and attenuated coronary perfusion perturbations (Berti et al., 2003). The beneficial effect of proanthocyanidins on coronary vasculature may be a result of stimulating synthesis of prostaglandinF_{1 α} and nitric oxide (Berti et al., 2003; Maffei Facino et al., 1996).

In an attempt to determine which part of polyphenols in red wine contributes to the well-known benefits of red wine, Fantinelli et al. (2005) segregated red wine polyphenols into four phenolic fractions consisting of polymeric proanthocyanidins, anthocyanins, flavonols and flavanols, and resveratrol, and added the fractions as well as the original red wine into the perfusate for 10 min before ischemia (20 min) and the first 10 min of reperfusion (30 min). Only proanthocyanidins exhibited the same protective properties as red wine, evidenced by restoration of left ventricular developed pressure, prevention of left

ventricular end diastolic pressure rise, low rate of lactate dehydrogenase release in the effluent, and diminished amount of lipid peroxidation.

2.9.6.3. Anthocyanins

Anthocyanins from black soybean seed coat orally administered in doses of 25, 50, and 100 mg/kg to rats 24 h before coronary artery occlusion for 30 min and reperfusion for 24 h reduced infarct size (Kim et al., 2006). Also, perfusing hearts for 30 min with cyanidin-3-glucopyranoside added to the perfusate following 30 min ischemia reduced production of malondialdehyde and preserved energy content (mainly ATP and NAD) in the myocardium (Amorini et al., 2003). Heart cells also showed a good uptake and accumulation of cyanidin. Furthermore, in a human study three months consumption of pomegranate juice showed improvement in myocardial perfusion and reduction in the risk of ischemia, as assessed by treadmill exercise or pharmacologic stress test (Sumner et al., 2005).

2.10. References

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CHAPTER 3

PROTECTION BY FLAVONOIDS AGAINST DEATH OF RAT EMBRYONIC VENTRICULAR (H9c2) CELLS INDUCED BY SIMULATED ISCHEMIA-REPERFUSION

3.1. Introduction

Ischemia or insufficient blood supply to the myocardium initiates a number of metabolic and ionic perturbations that can cause cell death and tissue necrosis. Reperfusion or restoration of blood circulation to the ischemic myocardium, although a necessary strategy to salvage myocardial cells from ischemic insult, paradoxically exacerbates the trauma that has already been caused by ischemia.

The pathological changes in heart tissue following ischemia and reperfusion mostly originate from deprivation of cells from oxygen. Insufficient oxygen results in cessation or deceleration of mitochondrial functions in particular oxidative phosphorylation that subsequently augments the electron leakage from the respiratory chain and increases generation of superoxide radicals (Powers et al., 2007). On the other hand, the impairment of oxidative phosphorylation is associated with suppression of the citric acid cycle and diminishing oxidation of fatty acids which are the major source of energy for heart cells (Solaini & Harris, 2005). Under such conditions, cardiac cells which are in constant demand for energy are compelled to activate anaerobic metabolism provided from the available glycogen stores. However, during ischemia cells have no or limited access to the blood stream, making it difficult to expel the extra lactate. This results in acidification of the intracellular milieu and consequently emergence of ionic disturbances such as elevation of sodium and calcium in the cytosol.

Severe disturbances in cellular homeostasis that occur during ischemia and reperfusion can terminate cell viability. Although recent notions have implicated myocardial cell renewal as a part of normal cardiac homeostasis (Nadal-Ginard et al., 2003) that can be of clinical importance after myocardial infarction (Beltrami et al., 2001), cardiac tissue likely

has limited ability to replace the dead cells and therefore prevention of cell death during ischemia-reperfusion bears therapeutic implications. Polyphenols and especially flavonoids are well acknowledged for their antioxidant and protective effect in circumstances of oxidative stress. They have also been recognized as potent anti-apoptotic agents. The aim of this study was to compare the effect of a selected group of polyphenols, mainly flavonoids, on cell death caused by a model of ischemia-reperfusion in cell culture using rat embryonic ventricular cells. While a few individual polyphenols have been examined for their ability to protect in models of heart ischemia-reperfusion in culture (Chang et al., 2007; Townsend et al., 2004), there has been no comparison of a range of different polyphenols to determine which compound/s is/are the most protective.

3.2. Materials and Methods

3.2.1. Cell culture

Rat embryonic ventricular myocardial cells, H9c2 (American Tissue Culture Collection, Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1.5 g/l NaHCO₃ and 1.0 g/l glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. Tissue culture flasks were kept at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed every 2-3 days and the cells were subcultured regularly. For experiments, cells were cultured on 24- or 96-well plates, kept in DMEM with 10% FBS until confluency and were switched to the DMEM with 2% FBS.

3.2.2. Treatment with polyphenols

Confluent cells were treated with various concentrations of polyphenols for various time courses prior to ischemia-reperfusion. Polyphenols were prepared in dimethyl sulfoxide (DMSO). Because DMSO is toxic to the cells, polyphenols were added to the medium in concentrated solutions to keep DMSO concentration equal to or less than 0.05% v/v.

3.2.3. Model of ischemia-reperfusion

Unless otherwise indicated, ischemia was induced by replacing the medium with ischemia-mimetic solution (described below) and reperfusion was established by re-introducing DMEM with 2% FBS to the cells. To remove polyphenols, cells including controls were washed twice with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.3) before the ischemia-reperfusion experiment. The cells in ischemia plates received ischemia-mimetic solution containing 140 mM NaCl, 1.25 mM CaCl₂, 1 mM MgCl₂, 8 mM KCl, and 6 mM HEPES, pH 6 (Maddaford et al., 1999). The concentration of potassium was higher and the pH was lower than physiological conditions to simulate the *in vivo* conditions during ischemia. The ischemia solution was bubbled with 100% nitrogen gas for at least 30 min before adding to the cells. The ischemia group was placed in a modular hypoxia chamber which was flushed with nitrogen gas for 1.5 h and kept sealed for the period of ischemia at 37°C. The time-course of flushing (1.5 h) was chosen according to the initial experiments when the atmosphere of the chamber was tested with the oxygen indicator methylene blue (Eigel et al., 2004). Methylene blue is a redox indicator which has blue colour in oxidizing environment (air) and turns colourless when reduced (by a reductant in the absence of oxygen). The period of ischemia was examined with relatively short (3-5.5 h) or long (9-10 h) episodes.

After ischemia, the ischemia solution was replaced with DMEM containing 300 mg/l NaHCO₃ and polyphenols (if any) and the plates were placed in the same chamber as the control plates. Control (non-ischemic) plates were given DMEM (with 2% FBS) containing 300 mg/l NaHCO₃ to keep pH at 7.4 in room atmosphere (with <0.1% CO₂), and kept at 37°C in a humidified chamber throughout the experiment. Those wells in the control plate which had been treated with polyphenols were treated the same during the experiment. Control and reperfusion plates were occasionally placed in separate chambers during the reperfusion time as the reperfusion chamber needed to be flushed with 100% oxygen for 15-20 min at the beginning of the reperfusion period.

3.2.4. Cell viability measurements

3.2.4.1. MTT reduction assessment

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) is a yellow coloured salt which is reduced by mitochondrial reductase enzymes of living cells to purple-coloured formazan crystals. The method used was a modification of the method used by Denizot & Lang (1986). Briefly, after reperfusion the medium was removed and the cells were washed twice with PBS to remove residual polyphenols. The medium was replaced with fresh reperfusion solution (140 mM NaCl, 1.25 mM CaCl₂, 1 mM MgCl₂, 6 mM KCl, 10 mM glucose, and 6 mM HEPES, pH 7.4 (Maddaford et al., 1999)), and MTT (in PBS) to a final concentration of 0.5 mg/ml (1.2 mM) was added and the cells were incubated for 1 h at 37°C. Then, the solution was removed, purple formazan crystals were solubilized in DMSO (100 µl for 96-well plates) for 15 min, and the absorbance was determined at 570 nm using an absorbance spectrophotometer.

3.2.4.2. Microscopic determination of cell viability

Acridine orange is a membrane-permeable fluorescent dye which interacts with DNA and RNA via electrostatic attraction, and can be used for staining both dead and viable cells. When bound to DNA, it fluoresces 525 nm light if excited at 502 nm. The cells were incubated for 1 h with a final concentration of 4 µg/ml (10.8 µM) acridine orange (in PBS) added to the medium (Vashishtha et al., 1998). After staining, the medium was aspirated, cells were washed with PBS, reperfusion solution containing glucose (described above) was added, and the cells were visualized using a fluorescence microscope. H9c2 cells are normally attached to the culture dish. When they die they are detached and therefore washed away during washing processes. Thus, the only cells observed on the microscope are live cells. However, because the cells are stained, unhealthy cells which still remain in the plate can easily be detected and excluded from counting. A specific area from each well with a magnification of 10X was chosen for imaging. A picture was taken and the number of cells in the field of view (3.1 mm²) was counted.

3.3. Statistics

Data represent means \pm SEM. Statistical analyses were carried out with SPSS software using one-way analysis of variance (ANOVA). As a post hoc test, 2-sided Dunnett was used where comparisons were with either control or ischemic-reperfused (named as IR in graphs) groups. In the absence of ischemia-reperfusion, polyphenol-treated groups were compared with the Control, while in ischemia-reperfusion conditions, polyphenol-treated groups were compared with the IR. Tukey's post-hoc test was applied where the comparisons were performed in the presence and absence of ascorbic acid. Adjustments were carried out where treatments with polyphenols and ascorbate increased cell viability in the control groups. The calculations were made based on the percentage of cell viability elevation in the control groups treated with antioxidants, and subtracting the same percentage from the corresponding antioxidant-treated group in the IR groups. A *p* value less than 0.05 was considered significant.

3.4. Results

3.4.1. Hypoxia contribution in cell death induced by ischemia-reperfusion

Since we aimed to compare the extent to which antioxidants, mainly polyphenols and ascorbic acid, alleviate the damage caused by oxidative stress in ischemia and reperfusion, the proportion of the harm produced by temporary hypoxia (lack of oxygen), relative to temporary ischemia (lack of oxygen, glucose, and serum), was evaluated (Figure 3.1). The hypoxia induced in our study produced moderate cell death in H9c2 cells after long (9-10 h) periods of ischemia, as about one-third of cell loss in the ischemia-reperfusion (IR) group was due to oxygen shortage and two thirds due to lack of FBS and glucose.

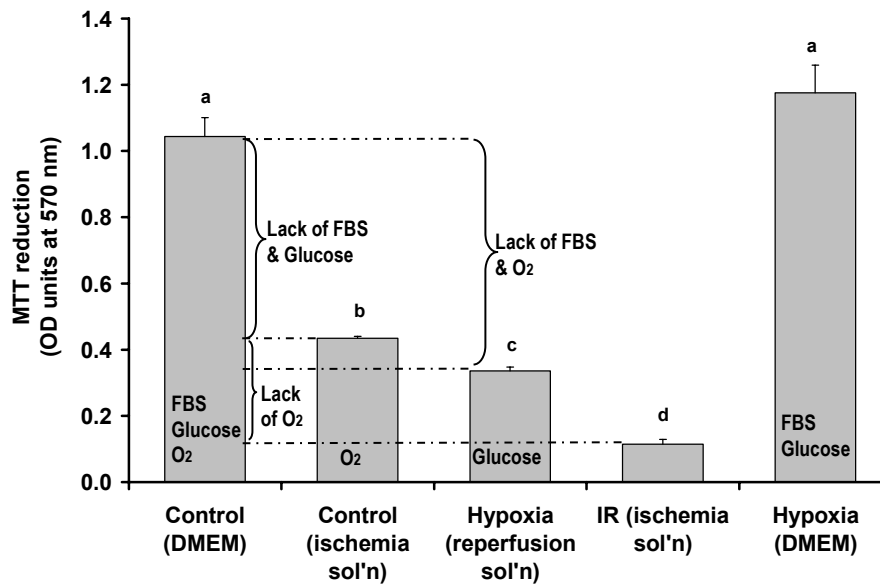


Figure 3.1. Contributions of oxygen, glucose, and serum removal on cell death after exposure to 10 h ischemia and 13 h reperfusion.

To determine the amount of cell death resulting from lack of oxygen, glucose, and FBS, cell viability was assessed in different experimental conditions using the MTT assay. The condition of the experiment for different groups was as follows:

Control (DMEM), cells in the control plate (access to oxygen) which received DMEM plus FBS throughout the experiment.

Control (ischemia sol'n), cells in the control plate (with oxygen) which received ischemia solution during the time of ischemia and DMEM plus FBS during the time of reperfusion.

Hypoxia (reperfusion sol'n), cells in the ischemia-reperfusion (IR) plate (flushed with nitrogen) which received reperfusion solution (containing glucose) during the time of hypoxia and DMEM plus FBS during the reperfusion time.

IR (ischemia sol'n), cells in the IR plate (flushed with nitrogen) which received ischemia solution during ischemia and DMEM plus FBS during reperfusion.

Hypoxia (DMEM), cells in the IR plate (flushed with nitrogen) which received DMEM plus FBS throughout the experiment. The ischemia and reperfusion time-courses were 10 and 13 h, respectively. Bars represent means of 3-6 wells \pm SEM. ^{a-d} Letters that are different indicate a significant difference at $p < 0.05$.

3.4.2. Testing polyphenols

3.4.2.1. Quercetin

With short-term (3-4 h) ischemia, quercetin exhibited either mild or no protection against ischemia-reperfusion. Treatment with 5 to 20 μM quercetin for 3 days prior to ischemia and during reperfusion could not significantly improve cell viability after 3 h ischemia (Figure 3.2). In control groups, 10 μM quercetin showed elevated MTT reduction that could be due to increased cell proliferation, while 40 μM quercetin showed some toxicity.

With 4 h ischemia and 5 μM quercetin the experiment was repeated 4 times (Figure 3.3). A slight (13%) but significant protection was observed under these conditions.

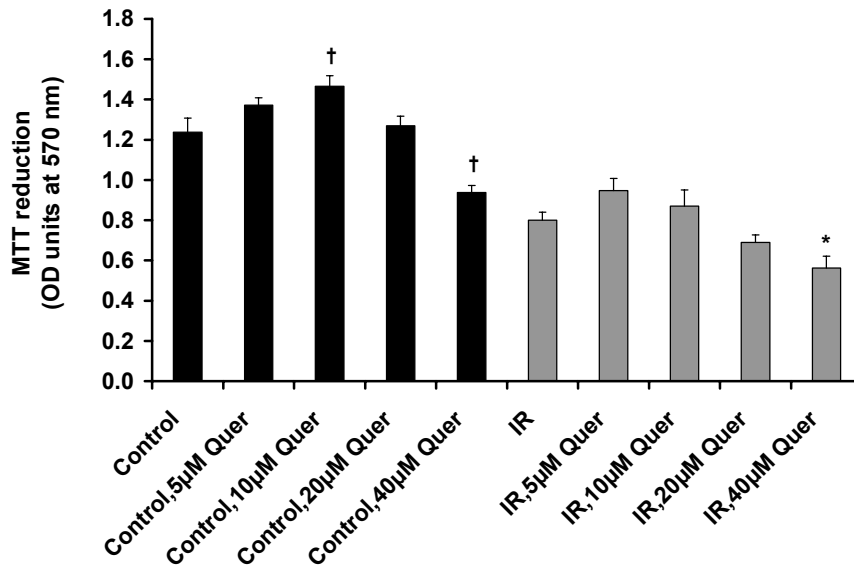


Figure 3.2. Cell viability after 3 h ischemia and 22 h reperfusion in cells pre-treated with quercetin (Quer) for 3 days.

Confluent cells were kept in 2% FBS-DMEM for 5 days including 3 days pre-treatment with different concentrations of quercetin, and then cells in the ischemia-reperfusion (IR) groups were exposed to ischemia for 3 h followed by reperfusion for 22 h. Quercetin was available to the cells during the reperfusion time. The number of live cells was detected by the MTT assay. Bars are the outcome of two independent experiments, each containing 5 wells. † is for $p < 0.05$ compared to Control. * is for $p < 0.05$ compared to the IR group.

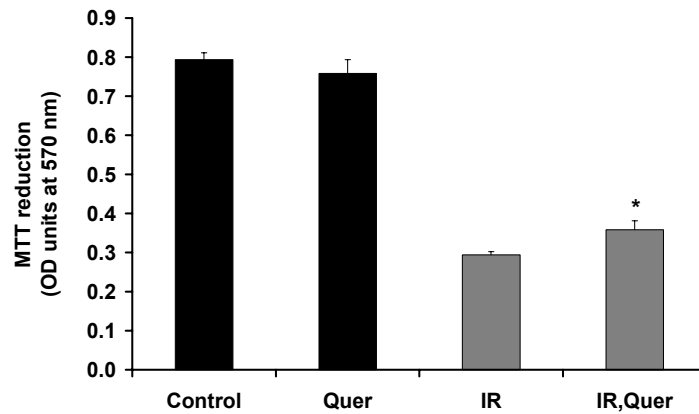


Figure 3.3. Cell viability after 4 h ischemia and 24 h reperfusion in cells pre-treated with quercetin (Quer) for 3 days.

Confluent cells were kept in 2% FBS-DMEM for 10 days including 3 days treatment with 5 μ M quercetin. Ischemia was established for 4 h followed by 24 h reperfusion. Quercetin was available to the cells during the reperfusion time. The number of live cells was detected with the MTT assay. Bars are means of four independent experiments, each assessing 4-5 wells. * is for $p < 0.05$ compared to the ischemia-reperfusion (IR) group.

With long (9-10 h) duration of ischemia, a similar situation was observed in quercetin-treated cells where quercetin exhibited either mild or no protection. In one experiment, concentrations of 0.1 to 15 μ M quercetin exhibited no protection after 9.5 h ischemia (Figure 3.4). In another experiment, 5 to 40 μ M quercetin increased cell viability by 18 to 33 percent after 9 h ischemia, while 100 μ M was cytotoxic (Figure 3.5).

Higher concentrations of quercetin showed cytotoxicity. At 40 μ M, quercetin exhibited a significant cytotoxicity in both control and IR-treated cells, causing 24 and 30 percent decreases in cell viability as assessed by MTT reduction, respectively (Figure 3.2). Similarly, at 100 μ M quercetin exacerbated cell death in both control and IR groups by 33 and 28 percent, respectively (Figure 3.5).

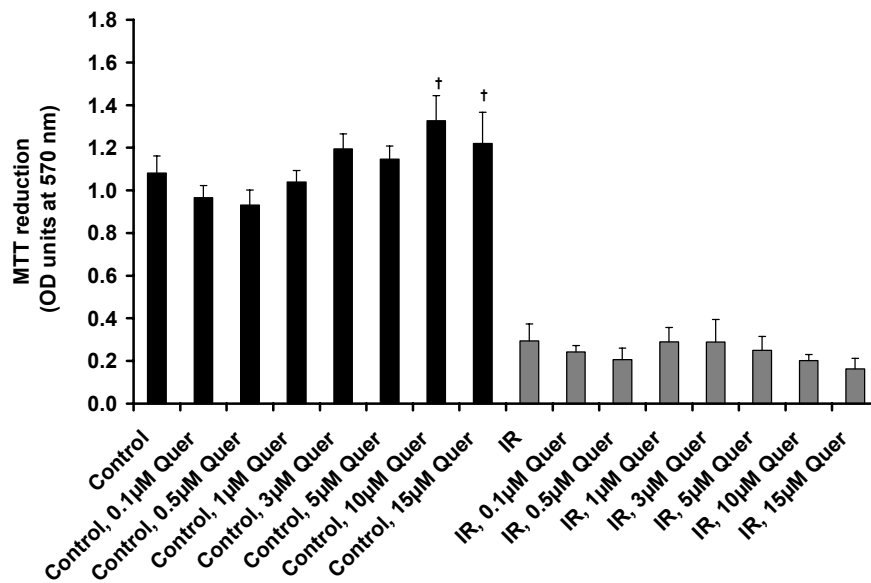


Figure 3.4. Cell viability after 9.5 h ischemia and 14 h reperfusion in cells pre-treated with quercetin (Quer) for 19 h.

Confluent cells were kept in DMEM with 2% FBS for 6 days before they were treated with various concentrations of quercetin for 19 h. Ischemia was induced for 9.5 h followed by reperfusion for 14 h. Quercetin was present during the reperfusion time. Cell viability was assessed by the MTT assay. Bars represent means of 4 wells \pm SEM. † is for $p < 0.05$ compared to Control. There was no difference between the ischemia-reperfusion (IR) groups before or after considering adjustments according to the controls.

Although was attempted to keep the concentration of DMSO, the solvent of polyphenols, in the DMEM to less than 0.05% v/v, it is yet possible that some of the toxicity of higher concentrations of polyphenols tested herein is in fact due to the higher level of DMSO rather than the higher concentration of polyphenols themselves.

Glutathione at 200 μ M did not exert any protective or damaging effect (Figure 3.5). It seems reasonable that the cells may not have been able to uptake this three-amino acid compound, and for this reason many researchers use the cell-permeable precursor N-acetyl cysteine (Borger & Essig, 1998).

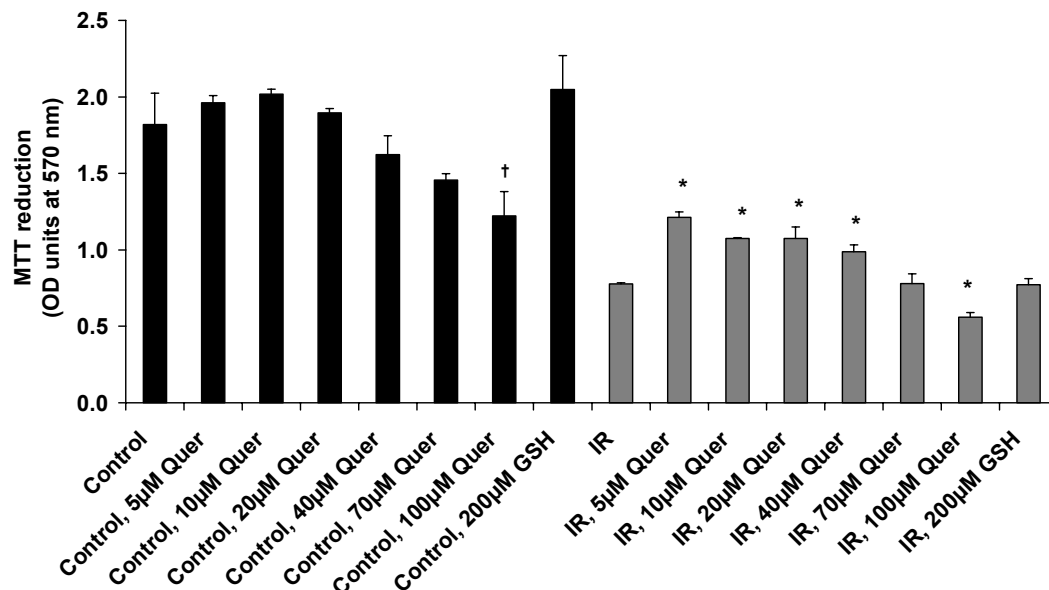


Figure 3.5. Cell viability after 9 h ischemia and 15 h reperfusion in cells pre-treated with quercetin (Quer) or glutathione (GSH) for 22 h.

Confluent cells were kept in DMEM with 2% FBS for 5 days before they treated with various concentrations of quercetin or 200 μ M glutathione for 22 h. Ischemia was induced for 9 h followed by reperfusion for 15 h. Quercetin and glutathione were accessible to the cells during the reperfusion time. Cell viability was assessed by the MTT assay. Bars are means of 4 wells \pm SEM. † shows $p < 0.05$ vs. Control. * shows $p < 0.05$ vs. ischemia-reperfusion (IR) with or without adjustments according to the corresponding controls.

3.4.2.2. Resveratrol

Increasing concentrations of resveratrol appeared to exhibit improvements in cell viability ranging from 12 to 25 percent, but none was statistically significant (Figure 3.6). In control groups, resveratrol at 5 and 10 μ M showed significant toxicity by 9 and 10 percent, respectively.

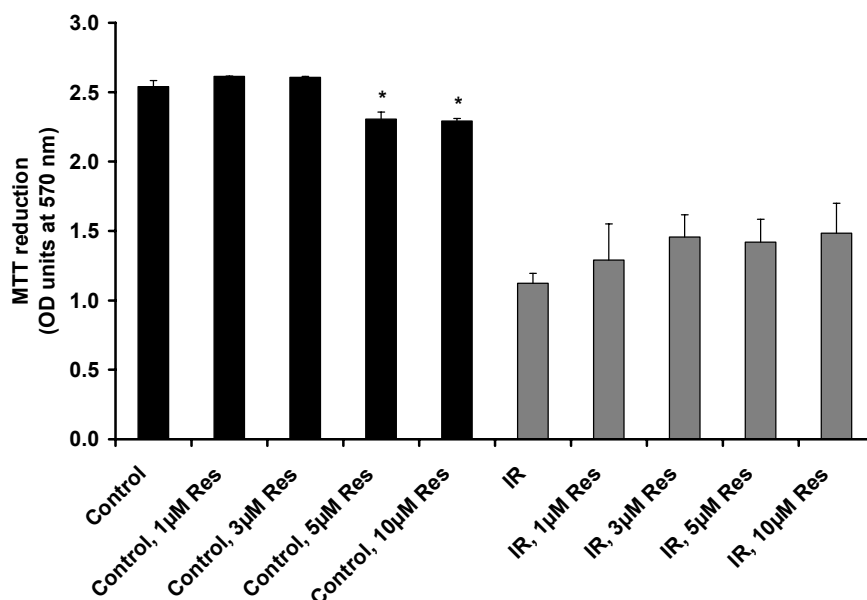


Figure 3.6. Cell viability after 9 h ischemia and 15 h reperfusion in cells pre-treated with resveratrol (Res) for 20 h.

Confluent cells were maintained in DMEM with 2% FBS for 6 days and incubated with various concentrations of resveratrol for 20 h. Resveratrol was present during the reperfusion time. Cells in the ischemia-reperfusion (IR) groups were subjected to 9 h ischemia and 15 h reperfusion. Cell viability was tested using the MTT assay. Bars indicate means of 4 wells \pm SEM. * shows $p < 0.05$ compared to Control. There was no significant difference between the IR groups.

3.4.2.3. Anthocyanins

Of flavonoids tested from the anthocyanin class, cyanidin did show significant but mild protection. Cyanidin in the concentration range of 3 to 40 μM increased cell viability assessed with MTT reduction by up to 16 percent, being statistically significant at 5 and 20 μM (Figure 3.7).

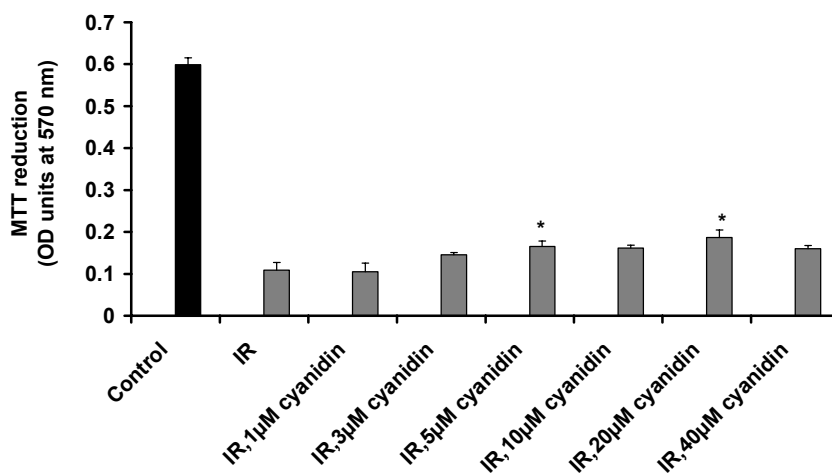


Figure 3.7. Cell viability after 9.5 h ischemia and 14 h reperfusion in cells pre-treated with cyanidin for 3 days.

Confluent cells were kept in DMEM with 2% FBS for 5 days including 3 days incubation with various concentrations of cyanidin before induction of ischemia for 9.5 h and reperfusion for 14 h. Cyanidin was present during the reperfusion time. Cell viability was measured by the MTT assay. Bars are means of 5 wells \pm SEM. * is for $p < 0.05$ vs. the ischemia-reperfusion (IR).

Delphinidin did not show any protection; instead, concentrations of 50 and 100 μM displayed toxicity (Figure 3.8). These concentrations caused 55 and 92 percent significant cell loss in control and 79 and 99 percent cell demise in the IR groups, respectively.

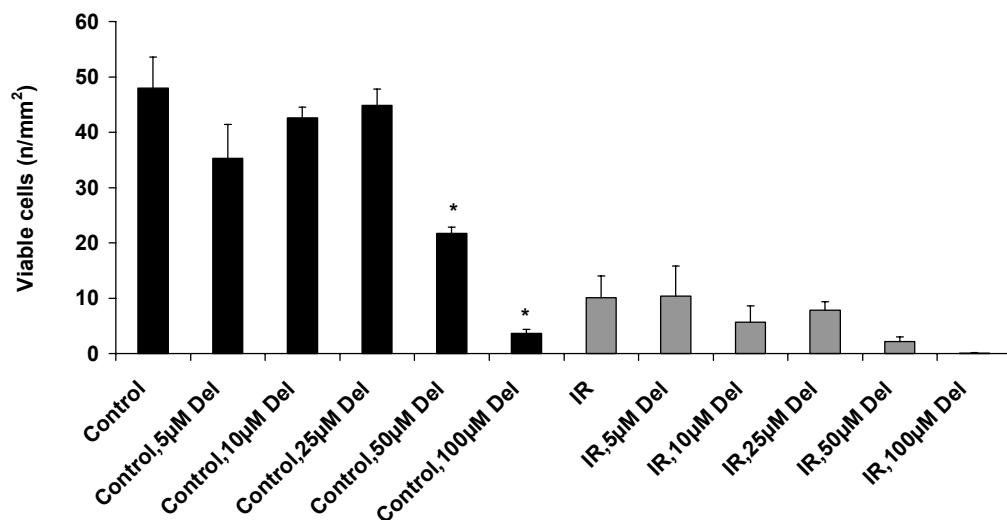


Figure 3.8. Cell viability after 5 h ischemia and 18 h reperfusion in cells pre-treated with delphinidin (Del) for 3 days.

Confluent cells were kept in 2% FBS-DMEM for 6 days including 3 days pre-treatment with various concentrations of delphinidin, and then ischemia-reperfusion (IR) groups were exposed to 5 h ischemia and 18 h reperfusion. Delphinidin was present during the reperfusion time. The number of live cells was counted after staining cells with acridine orange. Bars represent means of 3-4 wells + SEM. * shows $p < 0.05$ vs. Control. There was no significant difference between the IR groups.

3.4.2.4. Catechins

Catechins, including (+)-catechin, and (-)-epigallocatechin gallate and proanthocyanidins, which have a basic structure similar to catechin, exhibited good protection on the cells against damage caused by ischemia-reperfusion. In long-term pre-treatment, concentrations of 5 to 100 μM catechin improved cell viability by 21 to 58 percent, with concentrations of 5 to 50 μM being statistically significant (Figure 3.9). Similar to our results, Chang and colleagues (2007) found that 3 days pre-treatment with 25 μM catechin decreased chick cardiomyocyte death induced by ischemia-reperfusion by 22%.

Whereas in long-term (2-3 days) treatments concentrations from 5 to 50 μM catechin exhibited almost 50% protection (Figure 3.9 and 3.10), in short-term (1 h) treatment 50 μM catechin gave a 98% elevation in cell viability (Figure 3.10). Interestingly, no cytotoxic effect of catechin was observed in control and IR groups in either short- or long-term pre-treatments (Figures 3.9 and 3.10).

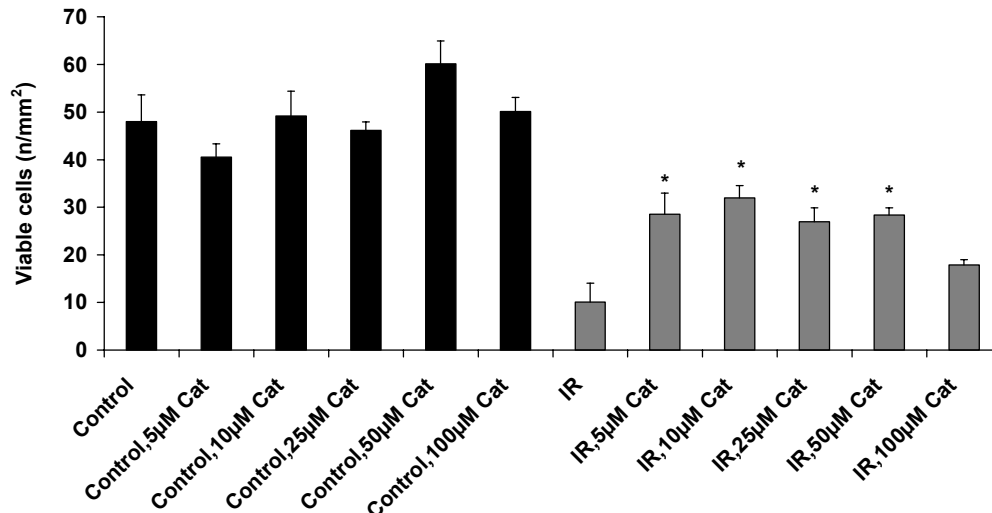


Figure 3.9. Cell viability after 5 h ischemia and 18 h reperfusion in cells pre-treated with catechin (Cat) for 3 days.

Confluent cells were kept in 2% FBS-DMEM for 6 days including 3 days pre-treatment with various concentrations of catechin, and then ischemia-reperfusion (IR) groups were exposed to 5 h ischemia followed by 18 h reperfusion. Catechin was present during the reperfusion time. The number of live cells was counted after staining cells with acridine orange. Bars represent means of 3-4 wells \pm SEM. * shows $p < 0.05$ vs. IR. No significant difference was present between control groups.

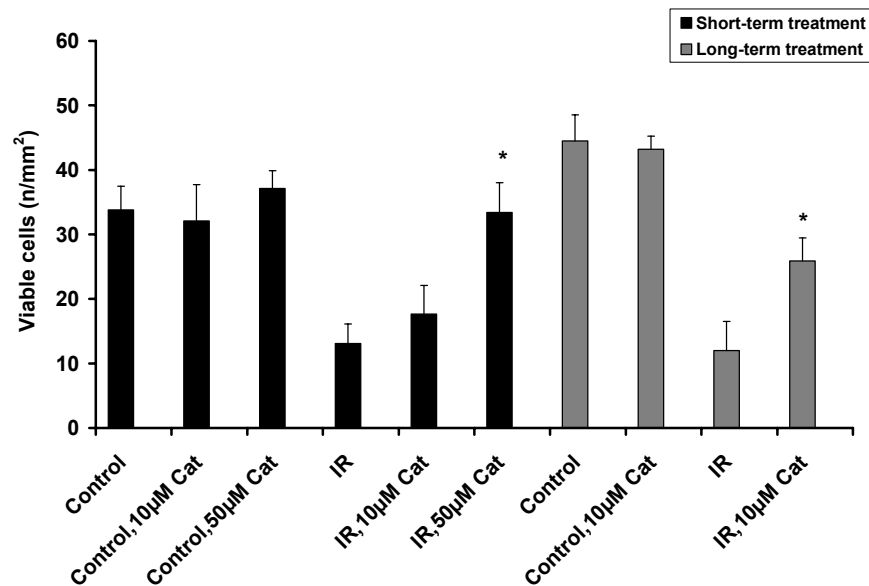


Figure 3.10. Cell viability after 5 h ischemia and 18 h reperfusion in cells pre-treated with catechin (Cat) for either 1 h or 2 days.

Cells were kept in DMEM with 2% FBS for 2 days and treated with either 10 or 50 µM catechin (Cat) for either 1 h (short-term) or 2 days (long-term) before establishment of ischemia for 5 h and reperfusion for 18 h. Catechin was present during the reperfusion time. Live cells were counted by a microscope after staining cells with acridine orange. Columns are means of 8 wells \pm SEM. * shows $p < 0.05$ compared to the corresponding ischemia-reperfusion (IR) group.

Concentrations of 5 to 100 μM epigallocatechin gallate and proanthocyanidins gave 28 to 49 and 22 to 44 percent protection, respectively, which in some concentrations were statistically significant (Figure 3.11). Consistent with our finding, Townsend et al. (2004) documented the anti-apoptotic effect of epigallocatechin gallate on neonatal primary cardiomyocytes against ischemia-reperfusion-induced cell death.

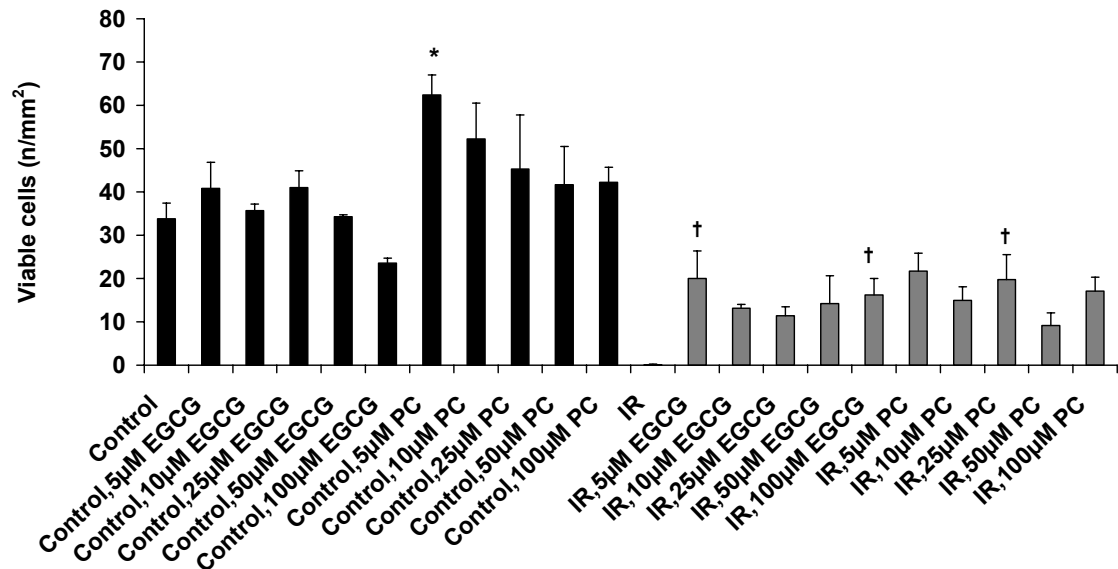


Figure 3.11. Cell viability after 4.5 h ischemia and 18 h reperfusion in cells pre-treated with epigallocatechin gallate (EGCG) or proanthocyanidins (PC) for 3 days.

Confluent cells were maintained in DMEM with 2% FBS for 6 days including 3 days incubation with various concentrations of epigallocatechin gallate or proanthocyanidins. The ischemia-reperfusion (IR) groups then were subjected to 4.5 h ischemia and 18 h reperfusion. Epigallocatechin gallate and proanthocyanidins were present during the reperfusion time. Viable cells were detected using a microscope following staining with acridine orange. Data are means of 3 wells \pm SEM. * indicates $p < 0.05$ vs. Control. † shows $p < 0.05$ vs. IR after considering adjustments according to the controls.

3.4.2.5. Ascorbic acid

A previous report (Guaquil et al., 2004) stated that dehydroascorbic acid is the form of vitamin C transported into both rat myocardial cells and rat primary cardiomyocytes, and after being transformed to ascorbic acid intracellularly can elicit protection against ischemia-reperfusion and hypoxia-reoxygenation. However, our results revealed the opposite in rat embryonic H9c2 cells. None of the concentrations of dehydroascorbic acid protected cells from ischemia-reperfusion (Figure 3.12), whereas sodium ascorbate gave remarkable protection at all concentrations tested, promoting cell viability by 57 to 92 percent (Figure 3.13).

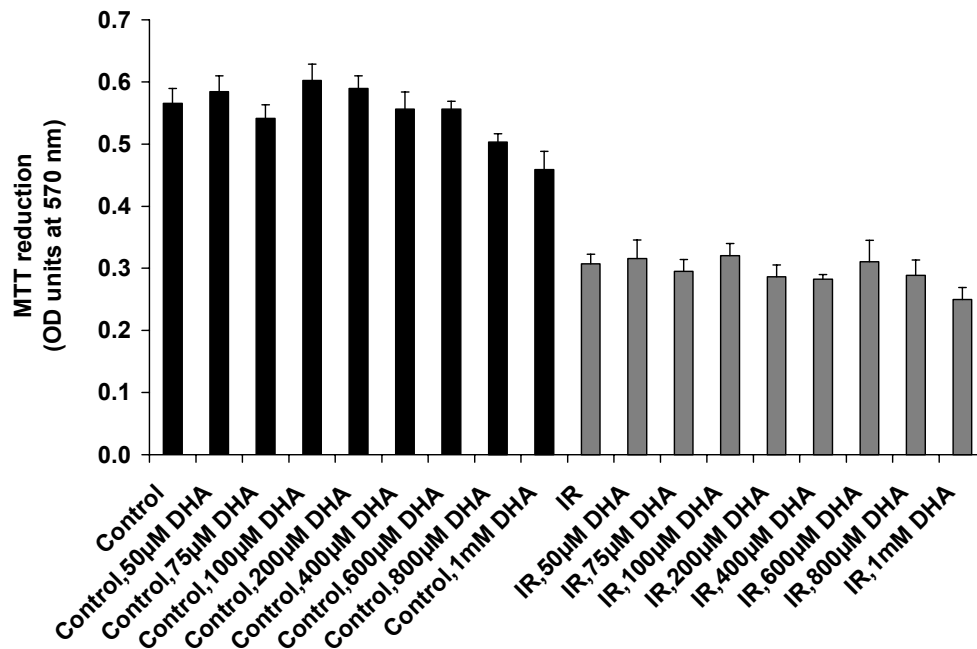


Figure 3.12. Cell viability after 9.5 h ischemia and 14 h reperfusion in cells pre-treated with dehydroascorbic acid (DHA) for 3 days.

Confluent cells were kept in 2% FBS-DMEM for 5 days including 3 days pre-treatment with dehydroascorbic acid. Then, ischemia-reperfusion (IR) groups were exposed to 9.5 h ischemia and 14 h reperfusion. dehydroascorbic acid was present during the reperfusion time. The MTT assay was used to measure viable cells. Bars show means of 5 wells \pm SEM. There was no significant difference between either control or IR groups.

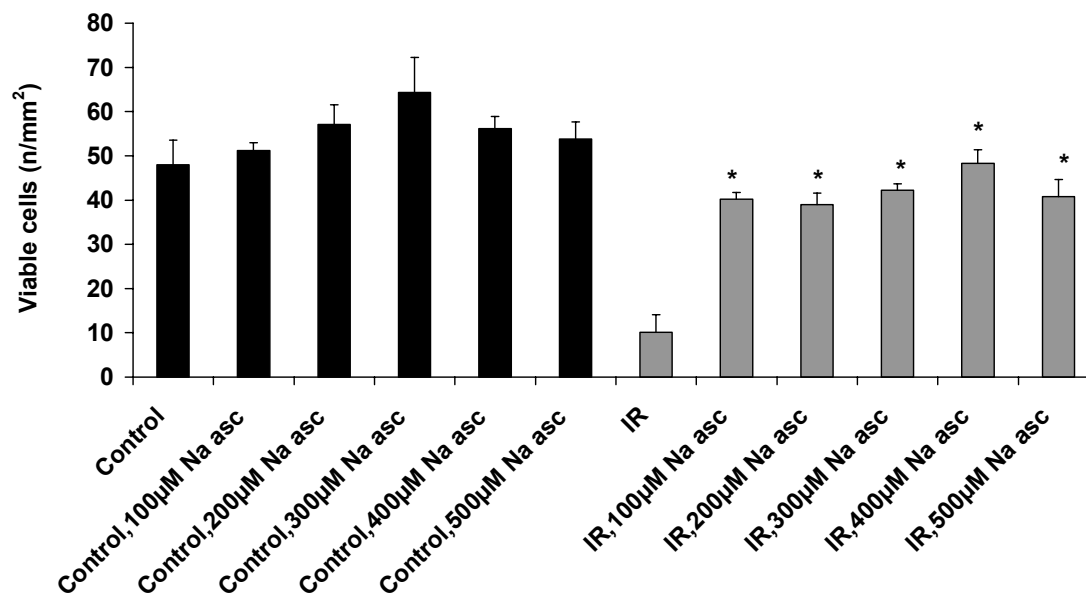


Figure 3.13. Cell viability after 5 h ischemia and 18 h reperfusion in cells pre-treated with sodium ascorbate (Na asc) for 3 days.

Confluent cells were maintained in 2% FBS-DMEM for 6 days including 3 days pre-treatment with various concentrations of sodium ascorbate, and then ischemia-reperfusion (IR) groups were exposed to 5 h ischemia followed by 18 h reperfusion. Sodium ascorbate was present during the reperfusion time. The number of live cells was counted after staining cells with acridine orange. Bars are means of 3-4 wells \pm SEM. * indicates $p < 0.05$ compared to the IR regardless if adjustments were or were not made. No significant difference was detected between the control groups.

3.4.2.6. Comparison between polyphenols in the presence and absence of ascorbic acid

In spite of protection that was observed by some polyphenols especially catechin, epigallocatechin gallate, proanthocyanidins, and ascorbic acid, a subsequent experiment revealed no protection by any of these and other polyphenols with or without the presence of ascorbic acid (Figure 3.14). The reason of this discrepancy is not clear, but in comparison with other experiments that showed protection by polyphenols and ascorbic acid this experiment had a lower difference between the Control and IR groups, and so the extent of oxidative stress may not have been sufficient to allow antioxidants to protect.

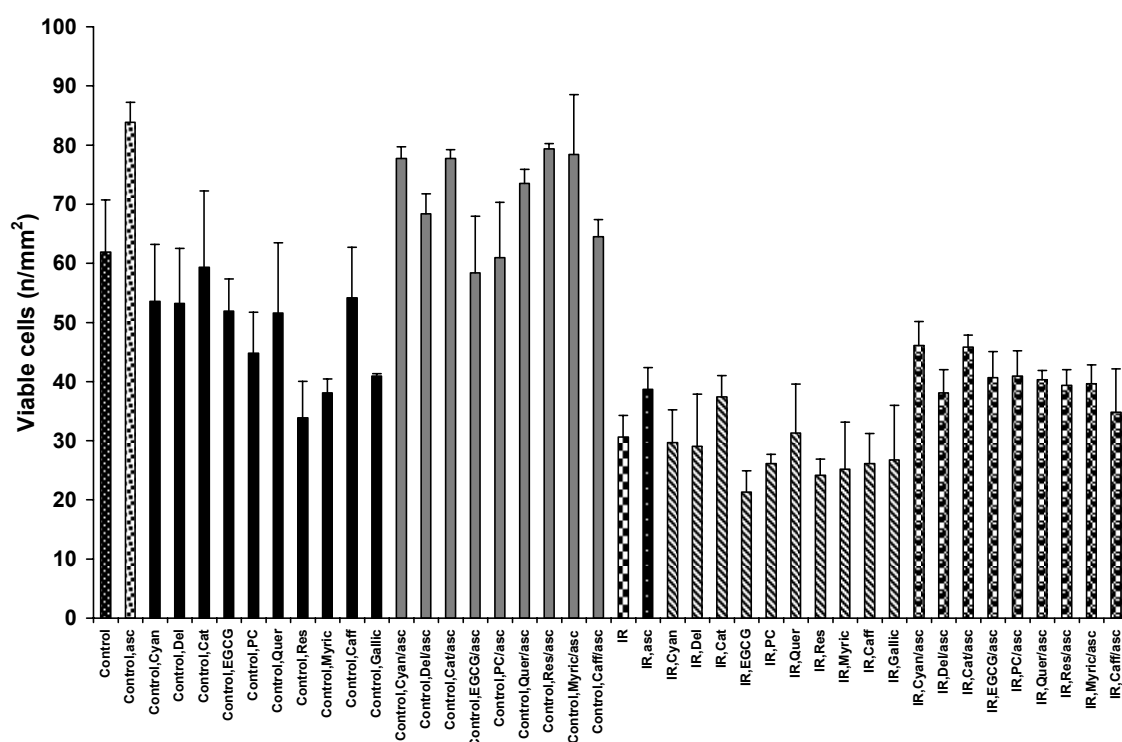


Figure 3.14. Cell viability after 5.5 h ischemia and 17 h reperfusion in cells pre-treated with a number of polyphenols in the presence or absence of sodium ascorbate (asc) for 3 days.

Confluent cells were maintained in DMEM with 2% FBS for 6 days including 3 days pre-treatment with either 300 μ M sodium ascorbate or 5 μ M of a polyphenol. Ischemia was induced for 5.5 h followed by reperfusion for 17 h. Cells were treated with the corresponding polyphenols with or without ascorbate during the reperfusion time. Viable cells were counted using a microscope after staining cells with acridine orange. Bars are means of 3-4 wells \pm SEM. No significant difference was observed between either control or ischemia-reperfusion (IR) groups either before or after performing adjustments. Cyan, cyanidin; Del, delphinidin; Cat, catechin; EGCG, epigallocatechin gallate; PC, proanthocyanidins; Quer, quercetin; Res, resveratrol; Myric, myricetin; Caff, caffeic acid; Gallic, gallic acid.

3.5. Discussion

The objective of these studies was to compare the effect of selected polyphenols, mainly flavonoids, on cell death caused by a model of ischemia-reperfusion in cultured H9c2 rat embryonic ventricular cells. Most of the cell death in this model was due to glucose and FBS removal, and merely one third of cells died due to hypoxia (Figure 3.1). However, oxidative stress may have been involved to a larger extent in the death of cells. It has been reported that serum withdrawal initiates apoptosis in a number of cell types (King et al., 2003; Maestre et al., 2003; Fernandez-Marinez et al., 2006; Voccoli et al., 2007). The process of death caused by serum removal involves mitochondrial dysfunction (Maestre et al., 2003) that leads to increased production of reactive oxygen species (Maestre et al., 2003; King et al., 2003), as well as depletion of endoplasmic reticulum Ca^{2+} stores (Voccoli et al., 2007) that results in cellular Ca^{2+} overload. These changes in Ca^{2+} and reactive oxygen species with serum withdrawal are similar to those that occur in *in vivo* models of ischemia-reperfusion. On the other hand, up to 76% of death after long ischemia has been due to FBS and oxygen deprivation and so to some degree involves oxidative stress, and the rest (24%) has been the result of glucose (and thus energy) deprivation (Figure 3.1). Interestingly, hypoxia alone did not decrease cell number (last bar in Figure 3.1). However, this does not necessarily mean that no cell death occurred in the hypoxic group, as with hypoxia both apoptosis and cell proliferation can be stimulated (Ray et al., 2008).

Quercetin in both short and long episodes of ischemia showed either mild or no protection (Figures 3.2 through 3.5). It is unclear why only in some, but not all, experiments quercetin showed protection. However, what is conspicuous is that protection, if any, by quercetin against cell death caused by the type of ischemia-reperfusion used in our study, was rather mild. Quercetin has been shown to induce phase 1 and more so phase 2 enzymes, including glutathione S-transferase, quinone reductase, glutamate cysteine ligase, and heme oxygenase, in 6 to 24 h incubation of rat primary cardiomyocytes with 30 μM quercetin (Angeloni et al., 2008). Whether quercetin also induces such enzymes in H9c2 cells, and whether the occasional mild protection exhibited by quercetin herein is partially due to this induction is not clear.

Similar to quercetin, resveratrol did not show a notable benefit. However, its toxicity merits attention, as it seemed to cause toxicity at lower concentrations compared to the other polyphenols tested herein (Figure 3.6). Nevertheless, the cytotoxicity of resveratrol was not

seen in the IR groups. This suggests that there may not be a serious concern for the toxicity of resveratrol in conditions of oxidative stress for which a benefit of resveratrol is validated.

The ability of resveratrol to induce antioxidants and phase 2 enzymes has been reported in H9c2 cells in 1 to 3 days incubation with 50 and 100 μ M resveratrol (Cao & Li, 2004). Moreover, resveratrol attenuated cell death and oxidative stress caused by xanthine oxidase and 4-hydroxyl-2-nonenal through a mechanism apart from its direct antioxidant effect. It is unlikely that resveratrol in our study has induced any of the antioxidant enzymes as both the concentrations and the incubation time used for resveratrol were too low to provide possibility of induction of phase 2 and antioxidant enzymes (Cao & Li, 2004). Surprisingly, the toxicity did not seem to be a concern in the study by Cao and Li. However, Leong et al. (2007) reported that resveratrol in concentrations ranging from 30 to 120 μ M inhibited cell proliferation and promoted differentiation in H9c2 cells without causing cell damage. Therefore, it is likely that the reduced cell viability in control cells treated with 5 and 10 μ M resveratrol in the current study has been in fact a result of inhibition of cell proliferation rather than causing cell injury. The mild reduction (5 to 10 percent) in cell viability at 5 and 10 μ M resveratrol confirms this speculation (Figure 3.6).

The cytotoxicity of delphinidin also deserves attention as it seemingly is more destructive than other polyphenols tested in our study (Figure 3.8). For instance, quercetin at toxic concentrations tested caused approximately 30% cell death in both control and IR groups (Figures 3.2 and 3.5), while delphinidin caused 55% cell death at the lowest toxic concentration tested (Figure 3.8).

We did not find considerable protection by cyanidin (Figure 3.7) and delphinidin (Figure 3.8) in H9c2 cells exposed to ischemia-reperfusion, except for mild (12 to 16 percent) protection with 5 and 20 μ M cyanidin. In this regard, our results are in contrast with those of other investigators who found a 30 to 40 percent decrease in H9c2 cell death induced by the anticancer agent, doxorubicin, after 24 h incubation with 50 and 100 μ M cyanidin and delphinidin (Choi et al., 2007). The discrepancy could be due to different mechanisms of cell death caused by doxorubicin and ischemia-reperfusion.

Of the polyphenols tested, catechins gave the most notable protection. Both long- and short-term treatments with catechin showed significant protection (Figures 3.9 and 3.10). Low concentrations (e.g. 10 μ M) of catechin seemed to work better if left with the cells for prolonged incubation times (in the range of days), while higher concentrations (e.g. 50 μ M) were capable of protection even over short-term (1 h) pre-treatment. The reason for such a

strong protection in short-term pre-treatment could be a direct antioxidant effect of catechin, as in long-term pre-treatments most of the catechin may have been oxidized during the regular cellular metabolism and due to factors such as transition metals present in the medium (Wee et al., 2003). If not much antioxidant activity remained after 3 days pre-treatment, why were such protections observed? The answer could be that catechins might have acted indirectly through signaling pathways to activate the antioxidant response element (ARE) and induce expression of antioxidant enzymes (Chen et al., 2000). Consistent with this supposition, Du and colleagues (2007) indicated that 24 h incubation of H9c2 cells with 50 μ M and more substantially 100 μ M catechin or proanthocyanidin B₄ increased activities of a number of cellular antioxidants and antioxidant enzymes and mitigated cell death caused by the xanthine oxidase system (Du et al., 2007).

Similar to catechin, epigallocatechin gallate and proanthocyanidins conferred considerable (up to 49%) protections (Figure 3.11). The protection exhibited by epigallocatechin gallate and proanthocyanidins is intriguing as the ischemia-reperfusion in the corresponding experiment was so intense that almost no cells remained in the IR group. This suggests that epigallocatechin gallate and proanthocyanidins may be appropriate flavonoids to work against severe states of oxidative stress. Furthermore, it has been reported that epigallocatechin gallate may be capable of inhibiting apoptosis induced by serum removal in cultured cells (Mandel et al., 2003), suggesting that this flavonoid may be capable of inhibiting or rectifying calcium disturbances following ischemia-reperfusion (Voccoli et al., 2007). Of note in the current study was that the protection by the catechins in long term pre-treatments (Figures 3.9 and 3.11) was dose-independent for the concentration range tested, suggesting protection by mechanisms other than direct antioxidant activities because one might expect increasing competition for ROS at increasing concentrations.

Although both epigallocatechin gallate and proanthocyanidins appeared to decrease the number of live cells at higher concentrations, no significant toxicity was detected (Figure 3.11). A pro-oxidant effect of proanthocyanidins has been reported for concentrations more than 100 μ M on chick cardiomyocytes (Shao et al., 2003).

The beneficial effect of ascorbic acid seen in our study (up to 92% protection with 3 days pre-treatment, Figure 3.13) is in accordance with previous observations from other investigators. For instance, ascorbic acid (100 μ M) improved cell survival by 40% in HL-1 mouse cardiac cell line subjected to hypoxia induced by cobalt chloride (Vassilopoulos & Papazafiri, 2005). A part of the ascorbic acid protection could be due to induction of phase 2

enzymes as the ability of ascorbic acid to induce phase 1 as well as phase 2 enzymes has been demonstrated in guinea pig liver after supplementing diets with ascorbic acid (Roomi et al., 1998). Ascorbic acid can auto-oxidize in the growth media (Wee et al., 2003) and a part of the observed protection could have been due to the mild oxidative stress produced by ascorbate auto-oxidation which might have induced phase 2 enzymes. In this way, pre-treatment with ascorbate may provide a form of preconditioning which can be clinically useful.

The toxicity of flavonoids may result from their pro-oxidant effect and oxidation products. As discussed earlier, as a part of their scavenging activity, flavonoids donate electrons and are concomitantly oxidized. The oxidized forms should be detoxified by other antioxidants, such as ascorbic acid (Blokhina et al., 2003) and glutathione (Galati et al., 2001), in order to be reduced or excreted in conjugated forms, respectively. If such detoxification is not performed, the oxidized forms of flavonoids can trigger oxidative reactions or react covalently with biomolecules (Galati & O'Brien, 2004).

On the other hand, flavonoids such as quercetin can reduce iron and copper and undergo metal-catalyzed oxidation to produce hydrogen peroxide and possible Fenton reactions (El Hajji et al., 2006). Since transition metals are sequestered by proteins *in vivo*, such reactions may not be of much concern in animal and human models, but they could be the underlying reason of toxicity exhibited by flavonoids in cell culture as both flavonoids and transition metals are together in the culture medium. Several flavonoids including catechin, epigallocatechin gallate, and quercetin have shown to undergo oxidation in cell culture media to produce hydrogen peroxide (Long et al., 2000). However, as catechins (Long et al., 2000) and ascorbate (Peterkofsky & Prather, 1977; Arakawa et al., 1994; Clément et al., 2001; Wee et al., 2003) have also been shown to produce hydrogen peroxide in culture medium, although mostly in concentrations much higher than what were used in the current study, and since catechins and ascorbate showed no toxicity in either control or ischemic-reperfused cells in our study, the involvement of hydrogen peroxide generated by metal-catalyzed oxidation of flavonoids can be ruled out. However, metal-catalyzed oxidation of flavonoids such as quercetin produces several degradation products (El Hajji et al., 2006) which may explain the cytotoxicity of flavonoids relative to ascorbate.

Further evidence that the cytotoxic effects of flavonoids may not have originated from reactive oxygen species generated during or after flavonoid oxidation are studies where antioxidants have failed to prevent cytotoxic and DNA-damaging effect of flavonoids

(Sasaki et al., 2007; Watjen et al., 2005). As an example, Sasaki and coworkers (2007) reported that quercetin at moderate concentrations (33 μ M) induced death in PC12 neuronal cell line, and N-acetylcysteine and glutathione could not inhibit its toxicity although they prevented hydrogen peroxide-induced cell death. Interestingly, quercetin did not protect the cells against hydrogen peroxide cytotoxicity but rather it exacerbated it.

Other possible explanations for flavonoid cytotoxicity could be perturbation in normal cellular metabolism including inhibition of enzymes such as protein kinases and DNA topoisomerases (Middleton et al., 2000). Spencer et al. (2003) reported that a neurotoxic effect of quercetin was mediated via inhibition of protein kinase B (Akt) and extracellular signal-regulated protein kinase (ERK). Moreover, inhibition of protein kinase C (Birt et al., 1996; Lin et al., 1997; Kang & Liang, 1997), mitogen-activated protein kinases (MAPKs) (Hou et al., 2004; Chen et al., 2004), and protein tyrosine kinases (Kandaswami et al., 2005; Lee et al., 2002; Kang & Liang, 1997) have been recognized among the mechanisms of chemopreventive, anti-proliferative and anti-tumor properties of flavonoids. The inhibitions are probably mediated through competition with ATP for binding to the active site of the enzymes (Lin et al., 1997; Stewart et al., 1999).

However, protein kinase signaling pathways possess many complexities. For instance, relatively low concentrations (25-50 μ M) of epigallocatechin gallate activated the apoptotic pathway in human breast cancer cells associated with elevation of cellular oxidative stress and activation of c-Jun N-terminal kinase (JNK), while higher concentrations (100-400 μ M) triggered necrosis without changes in cellular redox status or activation of JNK (Hsuuw & Chan, 2007). On the other hand, inhibition of protein kinases may contrarily confer benefits which could be of relevance to ischemia-reperfusion situations. For instance, it has been reported that inhibitions of MAPKs and nuclear factor-kappa B (NF- κ B) (Chae et al., 2007; Hong et al., 2007; Shin et al., 2007) and protein tyrosine kinases and serine/threonine kinases (including protein kinase B) (Maeda-Yamamoto et al., 2004) are involved in the anti-inflammatory and anti-allergic effects of epigallocatechin gallate and also delphinidin (Hou et al., 2005). Similar inhibition of protein kinase C and MAPKs has also been suggested as the mechanisms of anti-platelet activities of quercetin plus catechin (Pignatelli et al., 2006) and resveratrol (Yang et al., 2008). Therefore, with all of the intricacies around protein kinase signaling pathways it is hard to make any speculation on whether flavonoids in our study have interacted with such pathways, and whether the observed protection, absence of protection, or toxicity can be attributed to such interactions.

3.6. Conclusions

1. In this model of ischemia-reperfusion in H9c2 cells, cell death resulted from the combined lack of oxygen, glucose, and serum, and is most likely to have occurred through mechanisms associated with oxidative stress.

2. Ascorbic acid, catechin, epigallocatechin gallate, and proanthocyanidins were the most protective compounds against ischemia-reperfusion-induced cell death.

3. A low concentration (10 μ M) of catechin was more effective when incubated with the cells for a longer time (2 days), while a higher concentration (50 μ M) could exert benefit even after 1 h incubation.

4. Quercetin showed either mild or no protection. Resveratrol, cyanidin, and delphinidin displayed no protection.

5. Higher concentrations (e.g. 50-100 μ M) of some polyphenols including delphinidin, resveratrol, and quercetin in long-term treatments (1-3 days) exhibited toxicity. The toxicity was more pronounced in the control than in the ischemic-reperfused cells.

In conclusion, flavonoids from the catechin family and ascorbic acid may protect heart cells against ischemia-reperfusion. Direct antioxidant activity as well as induction of antioxidant and phase 2 enzymes may be implicated in the mechanisms of protection. Caution should be taken when using high concentrations of polyphenols, especially in long-term treatments and non-oxidative conditions.

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CHAPTER 4

PROTECTIVE EFFECT OF FLAVONOIDS ON RAT EMBRYONIC VENTRICULAR H9c2 CELLS EXPOSED TO HYDROPEROXIDES

4.1. Introduction

Reactive oxygen species (ROS) are forms of oxygen with more positive reduction potential and more reactivity than molecular oxygen. ROS are key factors in cell signaling pathways, working as second messengers for a wide range of activities including cell growth and apoptosis (Filomeni & Ciriolo, 2006). Targets of ROS in such pathways are redox-sensitive proteins such as protein tyrosine phosphatases, phospholipases, and calcium channels (Genestra, 2007). However, as the concentration of ROS increases in the cell the role that they play in regulation of signaling pathways shifts from a maintenance physiologic role to a pathologic deleterious action (Kamata & Hirata, 1999). In the pathologic mode of action, ROS impose cell oxidative stress which can lead to cell demise. Cells, however, possess an antioxidant network to help fight against oxidative stress. The network comprises enzymatic antioxidants such as superoxide dismutase and glutathione peroxidase and non-enzymatic antioxidants such as glutathione and thioredoxin. Nevertheless, when oxidative stress overwhelms the cellular antioxidant capacity, the cells will fall into a death program; a moderate oxidative stress may activate apoptosis whereas an extreme insult can block apoptosis and proceed to necrosis.

Polyphenols, especially flavonoids, have antioxidant activity. They have shown inhibitory effects against a variety of ROS including hydrogen peroxide (Dajas et al., 2003) and peroxynitrite (Sadeghipour et al., 2005). Other than a direct scavenging effect, numerous mechanisms have been suggested for the antioxidant potential of flavonoids, one of which is sequestration of transition metals such as iron (Morel et al., 1993). For example, a part of the protective effect of flavonoids against hydrogen peroxide may originate from this metal chelating activity (Melidou et al., 2005). Furthermore, the induction of antioxidant and phase 2 enzymes has recently been acknowledged as one of the indirect mechanisms of antioxidant activity of flavonoids (Angeloni et al., 2008; Moskaug et al., 2005). Flavonoids

may also render their antioxidant effect by reinforcement of other cellular antioxidants, either working in an orchestrated manner with them (Haramaki et al., 1998) or by being preferentially oxidized preserving lipid soluble antioxidants (Lotito & Fraga, 2000). The ability of polyphenols to reduce oxidative stress may be enhanced in the presence of ascorbic acid even when ascorbic acid or polyphenols alone affords no cytoprotection (Skaper et al., 1996; Ferroni et al., 2004; Chen et al., 2004). Moreover, flavonoids have been suggested to work synergistically with ascorbate to protect membranes (Bandy & Bechara, 2001).

Whereas many studies on polyphenols have investigated antitumor activities of these compounds, there are also many that have focused on their protective and anti-apoptotic capacities which are mainly exerted through their antioxidant activities. The goal of the current study was to compare the effects of various polyphenols, mostly flavonoids, on H9c2 cell viability after exposure to hydroperoxides, and to examine whether the presence of ascorbate could improve the protection.

4.2. Materials and Methods

4.2.1. Cell culture and treatments

Cells were cultured according to the protocol described in Chapter 3. In brief, H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1.5 g/l NaHCO₃ and 1.0 g/l glucose, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells grew in flasks containing DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. For experiments, cells were cultured on 96 well plates, kept in DMEM with 10% FBS until they became confluent, and they were then switched to DMEM with 2% FBS.

Cells were treated with polyphenols and/or ascorbate for either short (1-2 h) or prolonged (1-3 days) periods prior to addition of *tert*-butyl hydroperoxide (*t*-buOOH). The cells were then incubated in *t*-buOOH for 24 h followed by assessment of cell viability.

4.2.2. Cell viability measurements

Cell viability was measured by either reduction of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) or counting cells after staining with acridine orange as described in Chapter 3. For the MTT assay, after incubation with *t*-buOOH, cells were washed twice with phosphate-buffered saline (PBS), and MTT was added in a final concentration of 1.2 mM to a glucose-containing solution (140 mM NaCl, 1.25 mM CaCl₂, 1 mM MgCl₂, 6 mM KCl, 10 mM glucose, and 6 mM HEPES, pH 7.4) (Denizot & Land, 1986). After 1 h incubation at 37°C, the solution was removed and purple formazan crystals were solubilized in 100 µl DMSO for 15 min, and the absorbance was detected at 570 nm using an absorbance spectrophotometer.

For the acridine orange staining method, the cells were incubated for 1 h in a final concentration of 11 µM acridine orange added to the medium (Vashishtha et al., 1998). Then, the medium was removed, the cells were rinsed with PBS, the above-mentioned glucose-containing solution was added, and the cells were imaged using a fluorescence microscope.

The activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) released into the medium was used as another indication of cell death. GAPDH activity was measured using acella-TOX kit according to the manufacturer's instructions. GAPDH is an important enzyme in the glycolysis pathway that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate in the presence of the NAD⁺ cofactor and inorganic phosphate. In the acella-TOX kit, the activity of GAPDH is coupled to that of the enzyme 3-phosphoglyceric phosphokinase to produce ATP, which is detected via the luciferase/luciferin bioluminescence methodology.

4.3. Statistics

Data are means ± SEM. Statistical analyses were performed with SPSS software using one-way analysis of variance (ANOVA). As post hoc tests, the 2-sided Dunnett's test was used where comparisons were made between either control or *t*-buOOH groups where ascorbic acid was not included in the experiment, while Tukey's test was applied where the comparisons were assessed in the presence and absence of ascorbic acid. In the statistical analysis, adjustments were made where treatments with polyphenols and ascorbate increased

cell viability in the control groups. The adjustments were made by calculating the percentage of cell viability elevation in the control groups treated with antioxidants, and subtracting the same percentage from the corresponding antioxidant-treated group in the *t*-buOOH category. A *p* value less than 0.05 was considered significant.

4.4. Results

Consistent with the results from the ischemia-reperfusion experiments (Figure 3.11), epigallocatechin gallate showed protection against *t*-buOOH (Figure 4.1). Cells treated with 100 μ M epigallocatechin gallate for 1 h had 54% more survival than cells treated with *t*-buOOH alone, although a lower concentration of epigallocatechin gallate (50 μ M) was ineffective. Resveratrol at the concentrations examined gave no protection.

One hour treatment with 50 and 100 μ M epigallocatechin gallate and resveratrol did not cause any significant damage in the control groups (Figure 4.1), but the same concentrations over 3 days revealed strong cytotoxicity (Figure 4.2). After 3 days treatment with such high concentrations of epigallocatechin gallate and resveratrol there likely were not many cells remaining in the plate before the cells were treated with *t*-buOOH, and so no protection by the flavonoids could be seen. Cytotoxicity of epigallocatechin gallate seemed to be more potent than resveratrol as at 50 μ M for example it caused 237% more cell death than resveratrol (Figure 4.2).

In contrast to the ischemia-reperfusion experiments where flavonoids were more beneficial over 3-day rather than 1 day treatments (Chapter 3), in the *t*-buOOH experiments more benefit was observed in 1 h treatments, and 3-day treatments revealed cytotoxicity of some polyphenols (Figures 4.1 and 4.2).

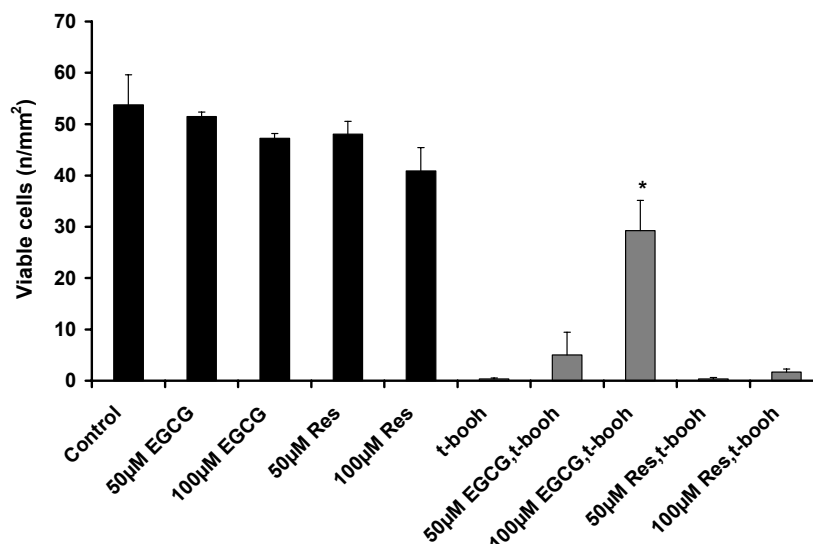


Figure 4.1. Effects on cell viability of epigallocatechin gallate (EGCG) and resveratrol (Res) added 1 h prior to *t*-buOOH.

Confluent cells were kept for 4 days in DMEM with 2% FBS before treating with 50 or 100 µM of epigallocatechin gallate or resveratrol for 1 h and then with 400 µM *t*-buOOH (shown as *t*-booh in the figure) for 24 h. Live cells were counted after staining with acridine orange. Bars are means of 3-4 wells ± SEM. * shows $p < 0.05$ vs. *t*-booh. There was no significant difference between the control groups.

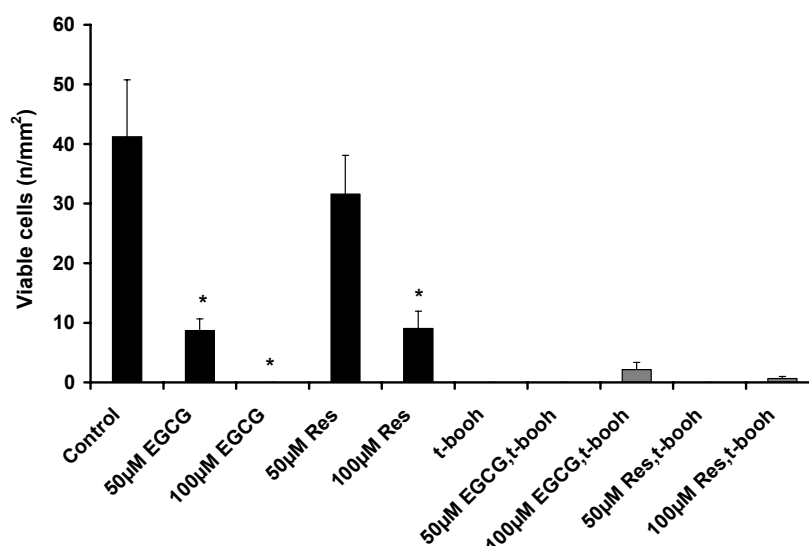


Figure 4.2. Effects on cell viability of epigallocatechin gallate (EGCG) and resveratrol (Res) added 3 days prior to *t*-buOOH.

Confluent cells were kept in DMEM with 2% FBS for 7 days including 3 days pre-treatment with either 50 or 100 µM of epigallocatechin gallate or resveratrol. Then, cells were incubated with 400 µM *t*-buOOH (shown as *t*-booh in the figure) for 24 h. Live cells were counted after staining with acridine orange. Bars are means of 3-4 wells ± SEM. * shows $p < 0.05$ vs. Control.

Catechin also showed a significant 28% protection at 100 μM added 1 h before *t*-buOOH, but lower concentrations (10 and 50 μM) did not exert significant protection (Figure 4.3). The ability of catechin to protect cells against *t*-buOOH was less than that of epigallocatechin gallate, as catechin at 10 and 50 μM concentrations increased cell survival by 6 and 6.5 percent (both non-significant), respectively, but epigallocatechin gallate at the same concentrations respectively produced 24 and 57 percent significant improvement in cell viability (Figure 4.3). The presence of 100 μM ascorbic acid lessened the cytoprotective effects of both flavonoids (Figure 4.3). Control groups treated with the same concentrations of catechin and epigallocatechin gallate with or without ascorbic acid were not adversely affected by the treatments.

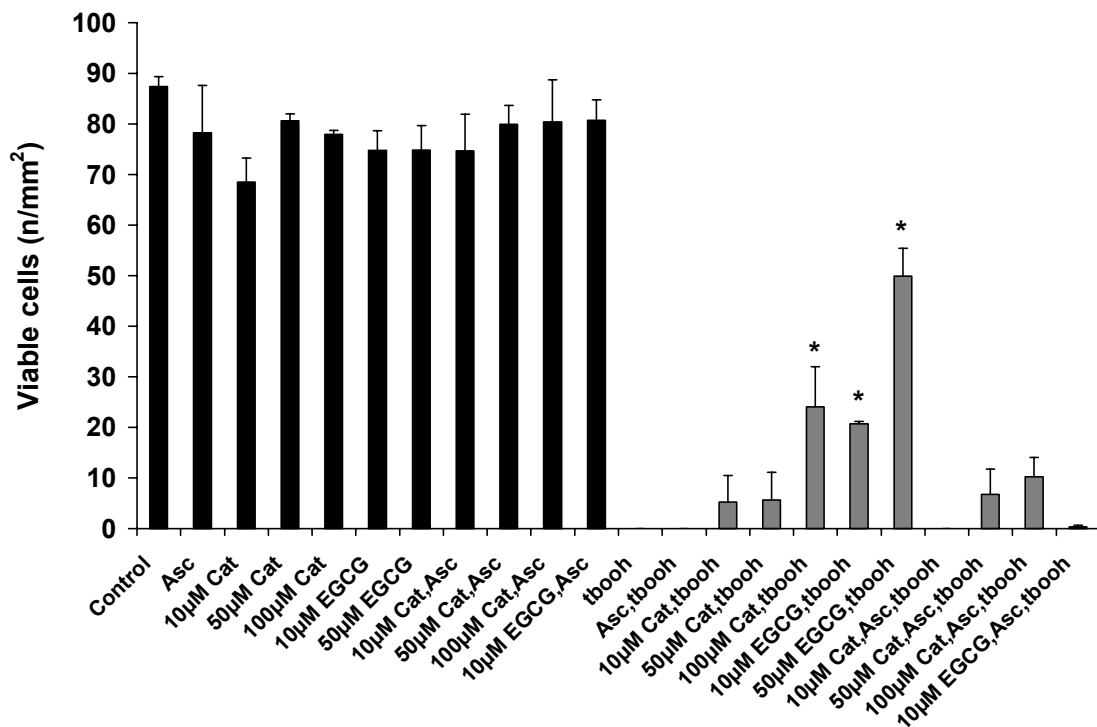


Figure 4.3. Effects on cell viability of catechin (Cat) and epigallocatechin gallate (EGCG) with or without ascorbate (Asc) added 1 h prior to *t*-buOOH.

Confluent cells were kept in DMEM with 2% FBS for 7 days and treated with different concentrations of catechin or epigallocatechin gallate alone or in combination with 100 μM sodium ascorbate for 1 h. Cells were then treated with 350 μM *t*-buOOH (shown as tbooh in the graph) for 24 h. Live cells were counted after staining with acridine orange. Data are means of 3-4 wells \pm SEM. † is for $p < 0.05$ vs. Control. * shows $p < 0.05$ vs. tbooh and Asc,tbooh. There was no significant difference between control groups.

Because higher concentrations of polyphenols were cytotoxic in 3-day treatments (Figure 4.1), lower concentrations (10 and 25 μM) were evaluated for their ability to protect and to cooperate with ascorbate. At 10 μM , none of polyphenols alone or in combination with ascorbate protected cells against *t*-buOOH (Figure 4.4-B). At this concentration, myricetin, epigallocatechin gallate + ascorbate, and quercetin + ascorbate revealed cytotoxic effects in the control groups (Figure 4.4-A).

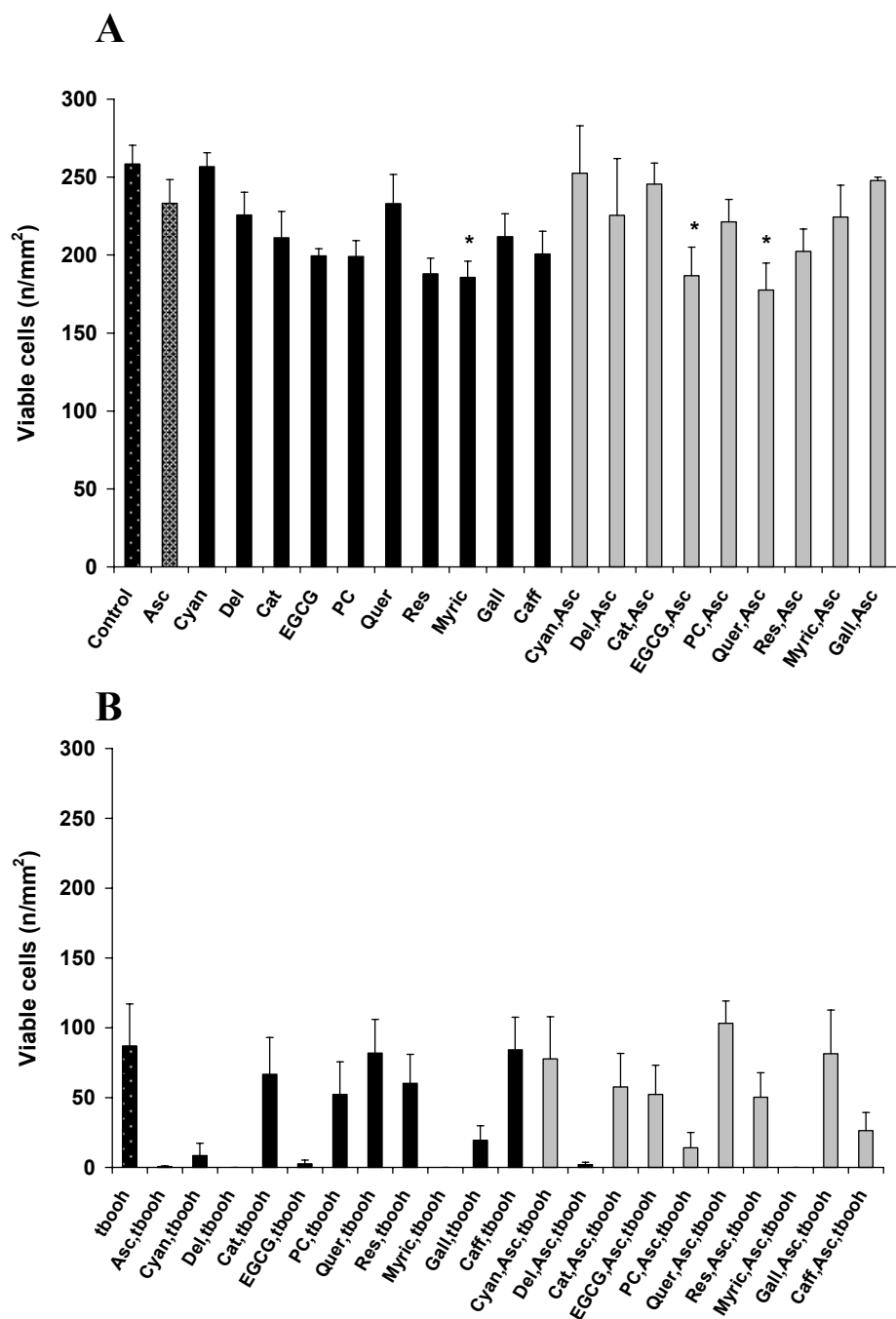


Figure 4.4. Effects on cell viability of 10 μ M of different polyphenols with or without ascorbate added 3 days prior to treatment with *t*-buOOH.

Confluent cells were kept in DMEM with 2% FBS for 5 days including 3 days pre-treatment with 10 μ M of polyphenols alone or in combination with 100 μ M sodium ascorbate. Then, cells were treated with 350 μ M *t*-buOOH (shown as *t*booh) for 24 h. Live cells were counted after staining with acridine orange. Asc, ascorbic acid; Cyan, cyanidin; Del, delphinidin; Cat, catechin; EGCG, epigallocatechin gallate; PC, proanthocyanidins; Quer, quercetin; Res, resveratrol; Myric, myricetin; Gall, gallic acid; Caff, caffeic acid. **A.** Cells in control groups (untreated with *t*-buOOH). Data are the mean of 3 wells \pm SEM. * indicates $p < 0.05$ vs. Control. **B.** Cells treated with *t*-buOOH. Bars are from two independent experiments each containing 4 wells. There was no significant difference between any of the conditions in the *t*-buOOH-treated cells.

Examination of 25 μM of the same compounds also did not show protection except for quercetin which revealed the same protection (39 to 46 percent) in the presence and absence of ascorbate (Figure 4.5). In contrast to the previous experiments, here ascorbate did not show a pro-oxidant effect in the presence of *t*-buOOH, although considering the required statistical adjustments according to the ascorbate-treated control group it also did not significantly protect cardiomyocytes against *t*-buOOH. The toxicity of polyphenols in *t*-buOOH-treated cells appeared more pronounced at 25 μM (Figure 4.5) compared to 10 μM (Figure 4.4). However, it was only quercetin which in combination with ascorbic acid significantly decreased cell viability in control groups. Nevertheless, the toxicity of quercetin in control cells implies that it should not be used for prolonged applications, and should be used only in conditions of oxidative stress.

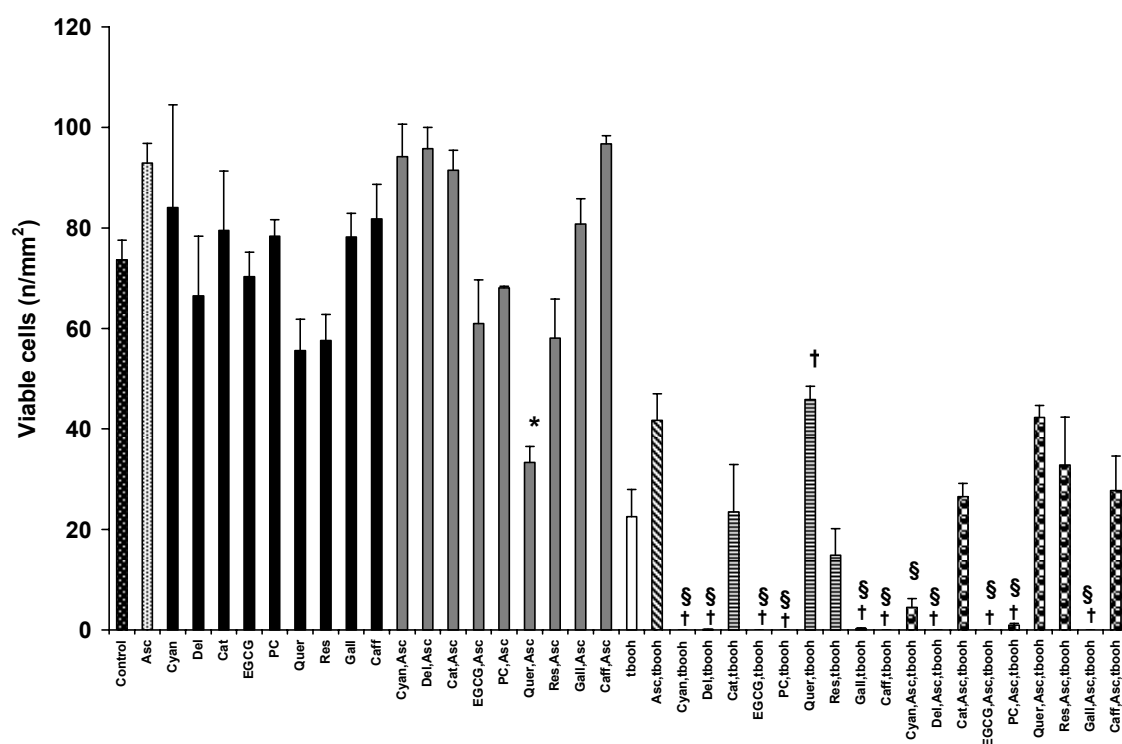


Figure 4.5. Effects on cell viability of 25 μM of different polyphenols with or without ascorbate added 3 days prior to treatment with *t*-buOOH.

Cells were maintained in DMEM with 2% FBS containing 25 μM of polyphenols alone or in combination with 100 μM sodium ascorbate for 3 days. Cells were then treated with 350 μM *t*-buOOH (shown as tbooh) for 24 h. Live cells were counted after staining with acridine orange. Columns are means of 3-4 wells \pm SEM. * shows $p < 0.05$ vs. Control. † indicates $p < 0.05$ vs. tbooh (with or without adjustments according to the corresponding controls). § indicates $p < 0.05$ vs. Asc,tbooh. Asc, ascorbic acid; Cyan, cyanidin; Del, delphinidin; Cat, catechin; EGCG, epigallocatechin gallate; PC, proanthocyanidins; Quer, quercetin; Res, resveratrol; Gall, gallic acid; Caff, caffeic acid.

To investigate whether any of the examined compounds is advantageous with short-term treatment, the pre-treatment time course was decreased to 1 h with 50 μ M of each polyphenol, with or without ascorbate. Epigallocatechin gallate and quercetin in the presence or absence of ascorbate were the only compounds that inhibited *t*-buOOH-induced cell death, increasing cell survival by 66 to 95 percent (Figure 4.6). Addition of ascorbate improved the cytoprotection exhibited by epigallocatechin gallate, while the quercetin effect did not change in the presence of ascorbate.

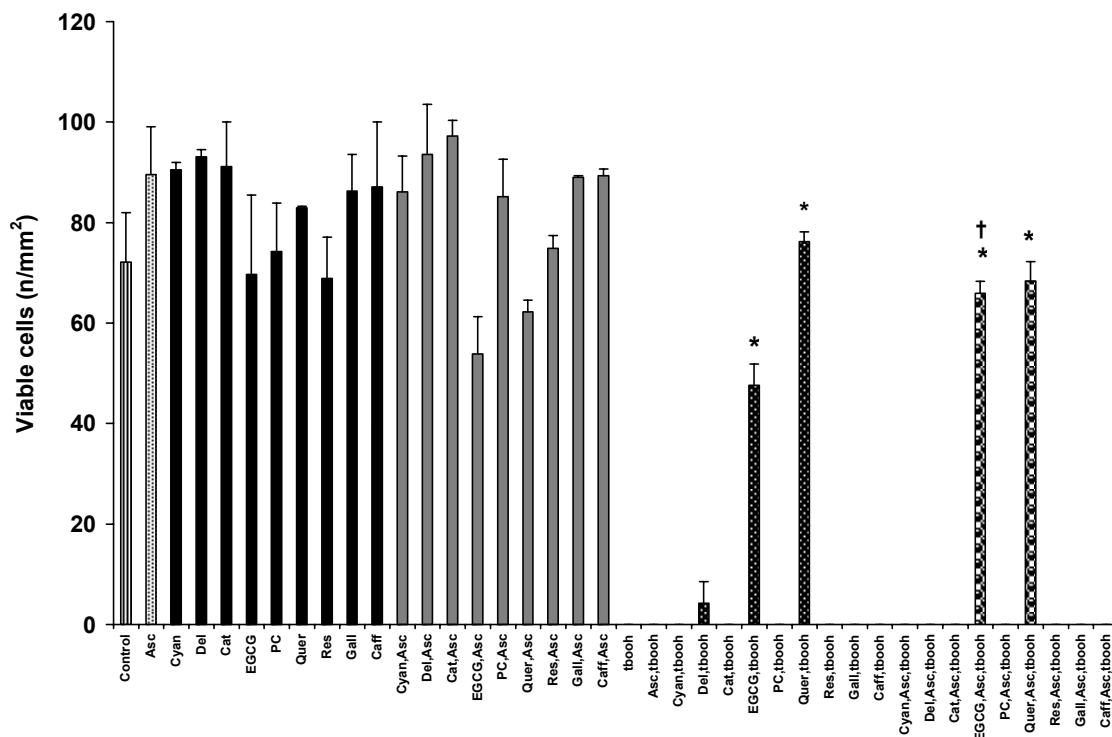


Figure 4.6. Effects on cell viability of different polyphenols with or without ascorbate added 1 h prior to treatment with *t*-buOOH.

Cells were kept for 2 days in DMEM with 2% FBS and treated with 50 μ M of polyphenols alone or in combination with 100 μ M sodium ascorbate for 1 h before treatment with 450 μ M *t*-buOOH (tbooh) for 24 h. Live cells were counted after staining with acridine orange. Bars show means of 3-4 wells \pm SEM. * shows $p < 0.05$ vs. tbooh (with or without adjustments according to the corresponding controls). † shows $p < 0.05$ vs. EGCG,tbooh. Asc, ascorbic acid; Cyan, cyanidin; Del, delphinidin; Cat, catechin; EGCG, epigallocatechin gallate; PC, proanthocyanidins; Quer, quercetin; Res, resveratrol; Gall, gallic acid; Caff, caffeic acid.

The protective effect of 50 μ M epigallocatechin gallate and quercetin with short-term pre-treatment was confirmed in a separate experiment which revealed 73 to 94 percent elevation in cell viability (Figure 4.7). Unlike in the previous experiment with epigallocatechin (Figure 4.6), no significant difference was observed in co-treatment of ascorbic acid with epigallocatechin gallate or quercetin. The supernatants of the same cells was used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) released from dead cells. The amount of GAPDH in the medium was increased by 240% in *t*-buOOH-treated cells (Figure 4.8). Ascorbic acid did not cause a significant reduction, but epigallocatechin gallate and quercetin gave significant declines of 77 and 124 percent in the absence of ascorbate, and 78 and 114 percent in the presence of ascorbate, respectively.

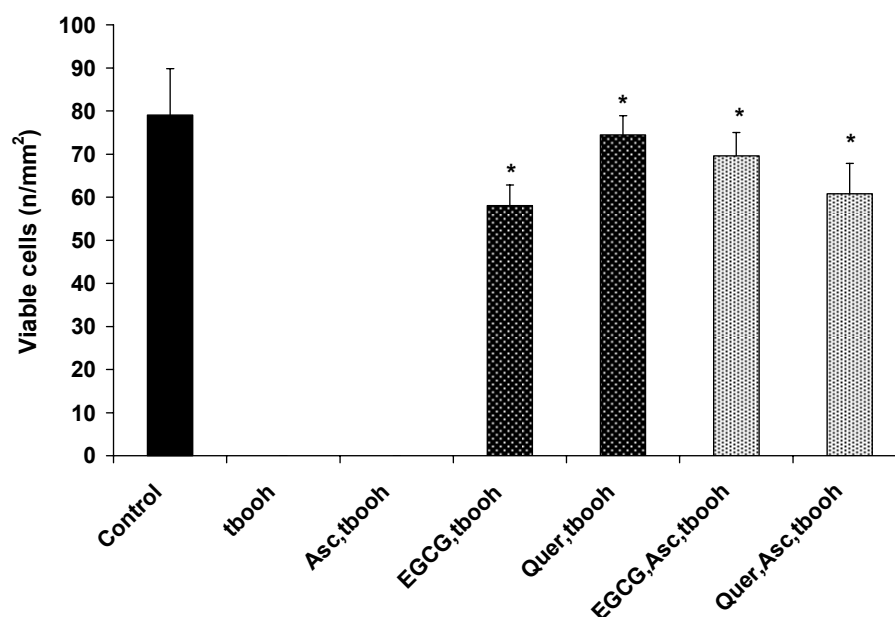


Figure 4.7. Effects on cell viability of epigallocatechin gallate (EGCG) and quercetin (Quer) with or without ascorbate added 1 h prior to *t*-buOOH.

Cells were treated with 50 μ M EGCG or Quer alone or combined with ascorbate (Asc) for 1 h before incubation with 450 μ M *t*-buOOH (tbooh) for 24 h. Viable cells were counted under a microscope after staining cells with acridine orange. Bars are means of 4 wells \pm SEM. * is for $p < 0.05$ vs. tbooh.

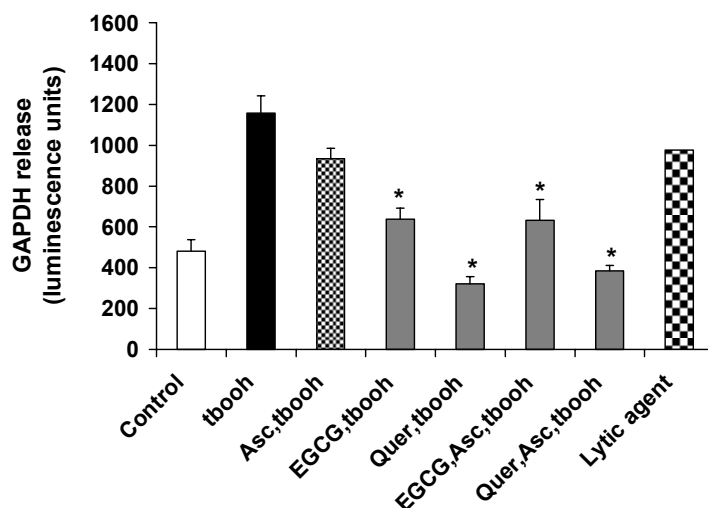


Figure 4.8. Effects on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) release of epigallocatechin gallate (EGCG) and quercetin (Quer) with or without ascorbate added 1 h prior to *t*-buOOH.

Cells were treated with 50 μ M EGCG or Quer alone or combined with ascorbate (Asc) for 1 h. Cell viability was tested by measuring glyceraldehyde diphosphate dehydrogenase in the supernatants of the cells 24 h after treating cells with 450 μ M *t*-buOOH (tbooh). Bars are means of 3-4 wells \pm SEM. * shows $p < 0.05$ vs. tbooh.

4.5. Discussion

The goal of this study was to compare the protective effects of polyphenols, alone and in combination with ascorbate, on H9c2 cells in conditions where cell death is more completely due to oxidative stress than was the case in the ischemia-reperfusion model. Compared to ischemia-reperfusion, in *t*-buOOH oxidative conditions long-term (3 days) pre-treatments with polyphenols exhibited less protection and more toxicity. In short-term (1 h) pre-treatments, 50 μ M quercetin and epigallocatechin gallate and less effectively 100 μ M catechin showed up to 95% protection. Ascorbate alone did not prevent the cytotoxicity of *t*-buOOH, and in combination with polyphenols, in most cases, did not improve the protection.

It is worthwhile to note that the micromolar concentrations of polyphenols administered in the current study are not achievable *in vivo* by means of a regular diet (Hertog et al., 1995), and therefore the amounts that are received by a regular diet may not induce a cytotoxic effect. Feeding with 50 mg/kg quercetin per day (that is higher than the average daily consumption of quercetin through a regular diet) for 11 weeks elevated plasma levels of quercetin in rats to 7.7 μM (de Boer et al., 2005). However, the concentration of quercetin in a variety of tissues was lower, with an average of 0.5 to 1 μM in tissues like liver, heart, kidney, and lung. In pigs, however, the concentrations of quercetin achieved in plasma and tissues were even lower (de Boer et al., 2005). Therefore, tissue achievable levels of flavonoids vary between different species.

Protection by quercetin and epigallocatechin gallate of cardiomyocytes against hydrogen peroxide has already been demonstrated. For instance, Park et al. (2003) found 50 to 87 percent improvement in H9c2 cell survival from 150 μM hydrogen peroxide following 30 min incubation with various concentrations of quercetin effective from 37 to 300 μM . Reduction of intracellular ROS, preservation of mitochondrial normal morphology, and induction of Bcl-2 proteins were among mechanisms of quercetin protection of H9c2 cells against hydrogen peroxide. Likewise, Choi et al. (2003) reported a 50% decrease in cell death from hydrogen peroxide following 30 min pre-incubation of human vascular endothelial cells with 50 μM epigallocatechin gallate or quercetin, while other flavonoids tested in their study were ineffective or damaging. Toschi et al. (2000) also found that 24 h treatment of neonatal rat cardiomyocytes with green tea extract prevented release of lactate dehydrogenase and formation of conjugated dienes during exposure to a free radical-generating system.

Regarding the best pre-treatment time-course for quercetin, our results are inconsistent with those from Angeloni et al. (2007) who reported 100% prevention of 150 μM hydrogen peroxide-induced death in H9c2 cells after 24 h pre-treatment with 30 μM quercetin, but not much protection after shorter incubation times (e.g. 18 h) with quercetin. The authors suggested induction of cellular antioxidants as one of the indirect mechanisms of protection displayed by quercetin (Angeloni et al., 2008). In contrast to them, we found the same magnitude of protection (92 to 94 percent) by quercetin (50 μM) after 1 h incubation (Figures 4.6 to 4.8), while with 3 days pre-treatment cell viability was improved by 46% with 25 μM quercetin (Figure 4.5) and showed no improvement with 10 μM quercetin (Figure 4.4). Although with a different cardiomyocyte cell type and using catechins, Dreger

et al. (2008), in agreement with us, reported that 1 h pre-treatment of rat neonatal cardiomyocytes with 50 μ M epigallocatechin gallate or 20 μ M theaflavin digallate inhibited cell death caused by 30 min incubation with 100 μ M hydrogen peroxide. They indicated that with increasing pre-treatment time the protective effects of the flavonoids decreased, suggesting the involvement of a direct antioxidant effect rather than indirect antioxidant induction. They documented that as a result of administration of epigallocatechin gallate, protein expression of heme oxygenase-1, but not glutathione peroxidase 3, superoxide dismutase 1 or catalase, was induced, but heme oxygenase-1 was not implicated in the cytoprotection exhibited by epigallocatechin gallate. They validated the involvement of a direct antioxidant effect of epigallocatechin gallate by observing reduction of intracellular ROS measured by dichlorofluorescein (Dreger et al., 2008).

In summary, the current study compared the ability of a number of different polyphenols and ascorbate to protect H9c2 cardiomyocytes against cytotoxicity of *t*-buOOH. Most other studies have not compared different polyphenols (and have used H₂O₂ instead of *t*-buOOH). Among the different polyphenols tested, only quercetin, epigallocatechin gallate and catechin were effective under the conditions tested, and only with short-term pre-treatment. This study was unable to confirm cooperation between ascorbate and flavonoids, as has been found previously in other systems (Skaper et al., 1996; Bandy & Bechara, 2001; Chen et al., 2004; Ferroni et al., 2004), except with epigallocatechin gallate. However the relative concentration of *t*-buOOH to the polyphenols may not have been optimal to detect this interaction.

4.6. Conclusions

In this model, unlike with ischemia-reperfusion (Chapter 3), 3 days pre-treatment with different polyphenols did not give protection and in many cases gave cytotoxicity. However, short-term (1 h) pre-treatment with some polyphenols gave protection. The best protection was observed in 1 h pre-treatment with 50 μ M quercetin followed by 50 μ M epigallocatechin gallate and 100 μ M catechin. Lower concentrations in the short-term were ineffective. Pre-treatment with ascorbic acid (100 μ M) did not prevent cytotoxicity of *t*-buOOH. Similarly, co-treatment of ascorbate with polyphenols in most cases did not improve cell survival upon treatment with *t*-buOOH, except in one case that ascorbic acid enhanced cytoprotection by epigallocatechin gallate.

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CHAPTER 5

PROTECTIVE EFFECTS OF SASKATOON BERRIES, GREEN TEA EXTRACT, AND BROCCOLI SPROUTS AGAINST ISCHEMIA-REPERFUSION INJURY IN ISOLATED RAT HEARTS

5.1. Introduction

Myocardial ischemia-reperfusion injury is the major cause of affliction, disability, and mortality after cardiac events such as heart attack and angioplasty. Following discontinuation of blood flow, delivery of nutrients and oxygen to the myocardial cells fails. As a result, mitochondrial oxidative phosphorylation is blocked but glycolysis primarily continues to supply energy (Solaini & Harris, 2005; Powers et al., 2007). Activation of anaerobic glycolysis is associated with production of lactate and consequently decline of intracellular pH, which initiates a succession of events that continues until reperfusion. By re-introduction of blood to the tissue at reperfusion, the already initiated ischemic damage is accelerated. In the first minutes of reperfusion, major changes in cytosolic and mitochondrial ionic concentrations occur, and substantial ROS are generated in the myocardial and surrounding non-myocardial cells, leading to activation of signaling pathways and eventually destruction of cells.

As elevation of ROS and the resultant oxidative stress is an important trigger of ischemia-reperfusion injury, one of the preventive interventions for this pathological circumstance is to reduce the magnitude of oxidative stress. One strategy could be administration of antioxidants such as superoxide dismutase and N-acetyl cysteine (Makazan et al., 2007). An alternative solution is to stimulate cells to produce more endogenous antioxidants, for example to express more superoxide dismutase (Tanaka et al., 2004). This enhancement may be achieved by activation of the Nrf2-ARE pathway which results in coordinated expression of phase 2 detoxification proteins, a group of proteins which are implicated in defence against xenobiotics and oxidative stress (Chen & Kunsch, 2004). The Nrf2-ARE pathway can be activated by various stimuli, among which are ROS and ischemia-reperfusion itself.

Flavonoids, a class of plant polyphenols for which health promoting properties have been appealing to many investigators for decades, have recently been identified for their potential to induce phase 2 enzymes, although they seem to work tissue and cell type-dependently (Moon et al., 2006). No study has yet attempted to investigate their phase 2 enzyme induction capacities in heart, especially under conditions of ischemia-reperfusion. As strong antioxidants, flavonoids might attenuate oxidative stress caused by ischemia-reperfusion, but they may also simultaneously stimulate Nrf2 and other possible transcription factors involved in the induction of cellular antioxidant proteins. Flavonoids also have other properties that are prophylactic for cardiovascular diseases such as anti-inflammatory, anti-apoptotic, and vasodilatory effects (Naruszewicz et al., 2007; Stangl et al., 2006). There are numerous reports available in the literature showing the protective effect of flavonoids against cardiac ischemia-reperfusion injury (Toufektsian et al., 2008; Ikizler et al., 2007; Bak et al., 2006).

Most of the interventions that are being suggested to alleviate heart ischemia-reperfusion injury are generally effective if introduced before ischemia (Bolli et al., 2004). However, introducing treatments before ischemia is sometimes hard to achieve especially in unexpected ischemic incidents such as myocardial infarction or heart arrest. Therefore, preferences are given to those interventions that are applicable in daily life. As an endogenous component of fruit and vegetables, flavonoids are present in our daily diet. By including plant materials rich in flavonoids in our daily diet, one can be prepared for possible unforeseen cardiac events. The objective of the present study was to investigate whether introducing flavonoid-rich foods in the diet can ameliorate biochemical indicators of ischemia-reperfusion injury.

Three feeding groups were included in our study. The diets were supplemented with either saskatoon berries, green tea extract, or broccoli sprouts. Berries were chosen as a source of anthocyanins (Clifford, 2000). They have been claimed to possess some of the highest amounts of antioxidants (Halvorsen et al., 2002) and phenolic acids (Mattila et al., 2006) among foods. Berries have shown the capability to reduce oxidative stress-induced cardiomyocyte cell death (Shao et al., 2004) and to protect cultured human endothelial cells against oxidative stress (Miranda-Rottmann et al., 2002). However, they have not been examined in heart ischemia-reperfusion. Jones et al. (1998) have tested the effects of Juniper berry oil on hepatic ischemia-reperfusion and observed a decrease in tissue damage and cell death, and improvement in hepatic microcirculation over a 2-week feeding period. There are

a few reports pointing to the effects of anthocyanins in heart ischemia-reperfusion. Toufektsian et al. (2008) fed rats with an anthocyanin-rich diet from maize kernels for 8 weeks and found higher levels of reduced glutathione in pre-ischemic hearts and lower infarct size in ischemic hearts. Kim et al. (2006) tested anthocyanins from black soybean seed coat and found reduced infarct size after oral administration of anthocyanin extract to rats 24 h before coronary artery occlusion. Amorini and colleagues (2003) reperfused isolated rat hearts with a synthetic derivative of cyanidin and reported decreased lipid peroxidation and preserved energy resources in treated myocardium. One aim of the present study was to examine whether feeding with berries in dietary achievable amounts could protect hearts against ischemia-reperfusion. This is important because the amount of anthocyanins consumed via food is generally far lower than their concentration when they are administered in a pure synthetic or extracted form.

A number of studies have already examined green tea extract or various forms of catechins. Townsend et al. (2004) administered green tea extract in drinking water to rats for one week before isolation of hearts for ischemia-reperfusion and observed reduced infarct size and apoptosis and improved cardiac function. The benefits of catechins on heart ischemia-reperfusion have also been demonstrated (Modun et al., 2003; Hirai et al., 2007; Aneja et al., 2004; van der Kraaij et al., 1989). However, the induction of phase 2 enzymes, as one of the possible mechanisms of catechin protection of heart against ischemia-reperfusion injury, has not been investigated.

Health benefits of broccoli sprouts have recently been claimed, and are postulated to be mainly due to isothiocyanates such as sulforaphane. Sulforaphane has been widely recognized as an anticancer agent by causing cell cycle arrest and apoptosis in cancer cells and as a chemopreventive agent through induction of phase 2 enzymes involved in detoxification mechanisms (Myzak & Dashwood, 2006; Zhang et al., 1992) and possibly inhibition of phase 1 enzymes implicated in activation of carcinogens (Myzak & Dashwood, 2006). It has been found that broccoli seeds, broccoli sprout extract, and sulforaphane induce the activities of quinone reductase, glutathione S-transferase, and glutamate cysteine ligase, and elevate intracellular glutathione levels in a number of cell and tissue types (Brooks et al., 2001; McWalter et al., 2004). No study has yet tested the effects of broccoli sprouts on hearts against ischemia-reperfusion injury.

The current study had two objectives. The first objective was to compare the effects of diets containing saskatoon berries, green tea extract, or broccoli sprouts on ischemia-reperfusion in isolated rat hearts. The second objective was to investigate if the effects were associated with alterations in phase 2 enzymes.

5.2. Materials and Methods

5.2.1. Animals, diets, and experimental groups

Animals were male Wistar rats weighing 250-300 g. All animals were maintained and handled under humane care in compliance with the national standards and guidelines of the Canadian Council on Animal Care (CCAC) and with approval of the University Committee on Animal Care and Supply (UCACS). The animals received food and water ad libitum until the start of the experiment (surgery). The animals were randomly assigned to one of the following feeding groups:

Sham (5 rats): AIN-93G diet

Control (8 rats): AIN-93G diet

Berries (6 rats): AIN-93G diet supplemented with 5% dried saskatoon berry

Tea (6 rats): AIN-93G diet supplemented with 0.25% green tea extract

Sprouts (10 rats): AIN-93G diet supplemented with 2% dried broccoli sprouts

The doses of saskatoon berries, green tea extract, and broccoli sprouts were chosen according to the previous works done by other investigators (Casto et al., 2002; Modun et al., 2003; Townsend et al., 2004; Wu et al., 2004b). The AIN-93G diet without *tert*-butylhydroquinone (an antioxidant and potential phase 2 enzyme inducer) was prepared in powder form and packaged under nitrogen by Dyets Inc. (Bethlehem, PA). The different experimental diets were prepared by mixing the powder form ingredients in a V-shell blender under 100% nitrogen and stored at -20°C until the time of serving to the animals. Saskatoon berries (a donation from Riverbend Plantations, Saskatoon, SK, Canada) were freeze-dried (final shelf temperature 40-50°C) for 48 h. Green tea extract was a commercial product called Polyphenon 60 (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) containing 63.9% of total catechins including 28.5% epigallocatechin gallate, 20.2%

epigallocatechin, 8.2% epicatechin gallate, and 7% epicatechin. Broccoli seeds (donated from Dr. Juurlink's laboratory, University of Saskatchewan) were of the calabrese cultivar which are high in glucoraphanin. Broccoli sprouts were grown for 6 days and air-dried for 1 week at room temperature.

All animal groups received the corresponding diets for 10 days before surgery. All the groups except sham were subjected to ischemia-reperfusion; hearts in the sham group underwent sham surgery (see below). The animals were weighed at the beginning of the 10-day feeding and before going for the surgery (Table 5.1).

5.2.2. Ischemia-reperfusion

The animals were anaesthetized with an initial flow of 5% and maintenance flow of 2.5% isoflurane in 1 L/min of 100% oxygen. The animals were first heparinized through the femoral vein with 350 IU/kg heparin, then the abdomen under the diaphragm was opened and the median lobe of the liver was excised and snap-frozen in liquid nitrogen. Then the chest was fully opened and the heart was removed, immediately immersed in cold perfusate, and cannulated through the aorta on a Langendorff apparatus in the quickest time possible. The hearts were perfused in the retrograde mode with modified Krebs-Henseleit buffer containing 118 mM NaCl, 1.2 mM KH_2PO_4 , 4.7 mM KCl, 1.7 mM CaCl_2 , 1.2 mM MgSO_4 , 20 mM sodium acetate, and 10 mM glucose, pH 7.4 (Imahashi et al., 1999). To remove any

Table 5.1. Weight of animals before and after 10 days feeding¹

	Before	After	Difference*
Sham	384.0±5.6	428.0±10.0	44.2±4.7
Control	368.5±5.3	418.0±9.5	49.5±7.0
Berries	372.7±2.7	419.5±6.1	46.8±5.5
Tea	371.0±2.9	418.2±6.7	47.2±4.5
Sprouts	373.3±1.8	418.3±4.3	41.0±4.7

¹ Data are the mean ± SEM.

* There was no significant difference between groups either before or after 10 days feeding, or in the weight gained.

particulate contaminants the buffer was filtered and kept at 2-8°C until the time of use. The perfusate was continuously bubbled with 100% oxygen, and warmed to 37°C using a heating coil connected to a circulating water bath. The temperature of the heart was maintained at 37°C by the warm buffer as well as a small water-jacketed chamber. The hearts were perfused at a constant pressure of 110 cm H₂O. A stabilization period of 15 min was used before ischemia. The initial flow rate during the stabilization period was set between 10-15 ml/min. After the period of stabilization, global ischemia was induced for 20 min by clamping one of the upper tubes of the apparatus at two places to ensure complete blockade of the perfusion, and then the perfusion was restored for 2 h by rapid unclamping of the above-mentioned tubes. At the end of reperfusion, the hearts were harvested, the weight of the whole heart and both ventricles was determined and the ventricles were snap-frozen in liquid nitrogen and kept at -70°C for later analysis. The hearts from the sham group were weighed and frozen at the end of the stabilization period.

5.2.3. Tissue crushing

Frozen ventricles were crushed to a powder using a tissue pulverizer prechilled in liquid nitrogen. For tissue analyses, weighed amounts of pulverized frozen tissues were homogenized in the appropriate buffer using a microcentrifuge tube homogenizer.

5.2.4. Assessment of protein content

Protein concentration was determined by the Bradford technique. Bradford reagent required for the assay was prepared using 100 ml of 85% phosphoric acid, 50 ml of 95% ethanol, and 100 mg of the dye. The solution was brought to 1 L with water, mixed thoroughly (left overnight while stirring), and filtered to remove undissolved dye particles. Generally, 5 µl of sample supernatants from tissues 10 times diluted with lysis buffer was added to 995 µl of the Bradford reagent in cuvettes. The absorbance of the dye was read at 590 and 450 nm, and the protein was quantified by dividing the absorbance at 590 nm by that at 450 nm (Zor & Selinger, 1996). To construct a protein standard curve, bovine serum albumin was used as the standard.

5.2.5. Flow rate and lactate dehydrogenase

To determine the coronary flow rate and lactate dehydrogenase released from the myocardial cells, heart effluents were collected for 1 minute at minutes 5 and 15 of the stabilization period, and minutes 1, 3, 5, 7, 10, 15, 20, 30, 60, 90, and 120 of the reperfusion time. For the sham hearts, the effluents were only collected from minutes 5 and 15 of the perfusion. Aliquots of the coronary effluents were kept refrigerated, and later in the day of the operation, 200 μ l of the effluent was mixed with 800 μ l of pyruvate solution containing 50 mM potassium phosphate, pH 7.4, 2.5 mM pyruvate, and 180 μ M NADH (added immediately before the assay), and the kinetics of the NADH consumption by the lactate dehydrogenase was recorded for 30 seconds at 340 nm. Lactate dehydrogenase activity was expressed as units of lactate dehydrogenase released into the perfusate/ml of coronary flow/wet weight of the heart.

5.2.6. Caspase-3 activity

Caspase-3 activity was assessed by incubation of tissue homogenates with a caspase-3 substrate and colorimetric detection of the cleaved substrate as a result of digestion by caspase-3 in cell lysates (Masini et al., 2003). Briefly, 30 mg of the frozen tissue was homogenized in 500 μ l cold lysis buffer containing 10 mM Hepes, 50 mM KCl, 5 mM MgCl_2 , 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin, pH 7.4. The homogenates were incubated on ice for 15 min, and centrifuged at 10,000 g for 10 min at 4°C. A 170 μ l sample of the supernatant was incubated with 6 μ l of 10 mM caspase-3 substrate Ac-DEVD-*p*-nitroanilide (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) (340 μ M final) for 1 h at 37°C, and the absorbance of the cleaved substrate, *p*-nitroanilide, was measured at 405 nm. The extinction coefficient of 8300 $\text{M}^{-1}\text{cm}^{-1}$ (Jacques et al., 2000) was used for determination of *p*-nitroanilide concentration. Data were expressed as μ mol of *p*-nitroanilide/min/mg of protein.

5.2.7. DNA fragmentation

DNA was extracted from the tissues and subjected to agarose gel electrophoresis (Fliss & Gattinger, 1996). About 100 mg of the frozen tissue was homogenized with 800 µl lysis buffer containing 10 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 8.0, incubated for 15 min and centrifuged at 13,000 g for 15 min, both at room temperature. From the upper part of the supernatant which contained fragmented DNA 450 µl was taken without disturbing the viscous lower part containing intact high molecular weight DNA. The supernatant was incubated with 100 µg/ml final concentration of proteinase K (EMD Chemicals, Inc., Gibbstown, NJ, USA) for 30 min at 50°C. To precipitate DNA, 60% final concentration of ethanol and 500 mM final concentration of NaCl were added to the proteinase-treated samples and the samples were left overnight at -20°C. To obtain DNA, samples were centrifuged at 13,000 g for 15 min at 4°C, and the pellet was resuspended in 500 µl TE buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Then, 500 µl phenol/chloroform saturated with TE buffer (1/1/2) was added, and the DNA was extracted by centrifugation at 20,000 g for 1 min at 4°C. The supernatant was treated with an equal volume of chloroform and again centrifuged at 20,000 g for 1 min at 4°C. The supernatant was taken and treated with final concentrations of 60% ethanol and 500 mM NaCl for 1 h at -20°C to precipitate DNA. DNA was obtained by centrifugation at 13,000 g for 15 min at 4°C and dried overnight at room temperature. Next day, the DNA was resuspended in 50 µl of TE buffer. To determine the concentration of DNA in the samples, a 10 µl aliquot of DNA in TE buffer was treated with a final concentration of 100 µg/ml RNase (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 30 min at 37°C, then 50 µg/ml final concentration of ethidium bromide was added and DNA concentration was determined by measuring ethidium bromide emission at 595 nm with excitation at 510 nm and using pure plasmid DNA as standard. Then, the rest of the samples were treated with a final concentration of 100 µg/ml RNase for 30 min at 37°C. An aliquot containing 2 µg of the RNase-treated DNA was mixed with 6X loading buffer containing 50% (v/v) glycerol, 50 mM EDTA, and 0.25% bromophenol blue, immediately loaded on a 1% agarose gel in TAE buffer (40 mM Tris-HCl, 30 mM acetic acid, and 2 mM EDTA, pH 8.0) containing 0.5 µg/ml ethidium bromide, and electrophoresed at 150 volts. *Hind* III-digested bacteria phage Lambda DNA was used as the standard DNA marker. The gels were then imaged with an Alpha Innotech digital imaging system, and the bands were densitometrically analyzed using

the AlphaEase (Alpha Innotech) software. The software measured the intensity of the fluorescence in the centre of the non-fragmented DNA band and of fragmented DNA at a fixed distance from this, and the data were expressed as the ratio of fragmented to non-fragmented DNA (Noda et al., 2001).

5.2.8. Thiobarbituric acid reactive substances (TBARS)

Peroxidation in tissues was measured by the TBARS assay based on the reaction of thiobarbituric acid with reactive aldehydes present in the samples, forming a colorimetric adduct that could be detected spectrophotometrically (Ohkawa et al., 1979). An approximate amount of 50 mg of the frozen tissues was homogenized with 200 µl of RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 0.0002% butylated hydroxytoluene (BHT), 1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin, pH 7.4. The homogenates were incubated for 30 min at room temperature, and centrifuged at 15,000 g for 10 min at 4°C. The protein content was measured and 100 µl of the supernatant was added to 350 µl of thiobarbituric acid solution containing 12% acetic acid, pH 3.5, 0.6% SDS, 0.45% thiobarbituric acid, and 0.0002% BHT, and heated for 1 h at 95°C. The heated samples were then cooled, centrifuged at 4,000 g for 10 min, and the absorbance of the supernatant was measured at 532 nm. The concentration of malondialdehyde (MDA) was calculated using a standard curve of malondialdehyde prepared from tetraethoxypropane by acid hydrolysis (Esterbauer & Cheeseman, 1990). The amount of MDA in samples was expressed as nmol/mg of protein.

5.2.9. Aconitase activity

Aconitase activity was evaluated by measuring the amount of *cis*-aconitate produced from either citrate or isocitrate (Takemoto et al., 2001). About 20 mg of the frozen tissues was homogenized with 200 µl of lysis buffer containing 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 1 mM cysteine, 1 mM citrate, 0.5 mM MnCl₂, and 1 mM PMSF, incubated for 15 min at room temperature, and centrifuged at 15,000 g for 10 min at 4°C. Then, 20 µl of the supernatant (~ 15 µg of protein) was added to quartz cuvettes with 780 µl water and 200 µl aconitase reaction buffer containing 50 mM Tris-HCl, pH 7.4, 20 mM isocitrate, and

0.5 mM MnCl₂, incubated for 2 min, and the absorbance of *cis*-aconitate was measured at 240 nm. The amount of aconitase in the samples was determined using a standard curve for *cis*-aconitate and the aconitase activity was reported as μ moles of *cis*-aconitate produced/min/mg of protein.

5.2.10. Glutathione measurements

Tissue glutathione and glutathione disulfide were determined using a method described by Tietze (1969). To measure glutathione, 30 mg of tissue was mixed with 300 μ l meta-phosphoric acid solution containing 1% meta-phosphoric acid. Samples were left on ice for 15 min and centrifuged at 10,000 g for 15 min. For glutathione (GSH) measurements, 40 μ l of the protein-precipitated supernatants was added to 160 μ l of 50 mM potassium phosphate buffer, pH 11, mixed and the background absorbance was measured at 412 nm. Then, 10 μ l of 5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (in methanol) (250 μ M final) was added, mixed and incubated for 20 min in the dark. A second reading at 412 nm was performed and the difference between the two readings was used for determining the glutathione level in the tissue.

For glutathione disulfide (GSSG) measurement, 40 μ l of the protein free supernatants was added to 160 μ l of 50 mM potassium phosphate buffer, pH 11, with 8 μ l of 5 mM NADPH (200 μ M final). The initial absorbance at 340 nm was recorded, and 5 μ l of 20 U/ml glutathione reductase (0.5 U/ml final) was added, mixed, and the samples were incubated at room temperature for 40 min and read again. The difference of the first and the second reading indicates NADPH consumption, and therefore the amount of glutathione disulfide present. Standard curves were plotted using pure glutathione and glutathione disulfide. The values for tissue glutathione were calculated according to the standard curves and weight of the tissues used.

For measuring glutathione content in liver tissues, more diluted samples were used. For protein precipitation, 400 μ l of 3% meta-phosphoric acid solution was used for 30 mg of liver tissues. After centrifugation, 20 μ l of the supernatants was used for both glutathione and glutathione disulfide measurements.

5.2.11. Heme oxygenase-1 protein level

To determine heme oxygenase-1 protein level in heart, 30 mg of the tissue was homogenized in 300 µl RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM DL-dithiothreitol (DTT), 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin, pH 7.4. The homogenates were incubated for 20 min on ice, and centrifuged at 15,000 g for 10 min at 4°C. The protein content was measured, and homogenates were denatured in sample loading buffer (100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 2% SDS, 15% glycerol, 0.03% bromophenol blue (in methanol), 2% β-mercaptoethanol) at 95°C for 10 min (Tron et al., 2005). Aliquots containing 60 µg of total protein were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% resolving gel. Proteins were transferred onto nitrocellulose membranes using a semi-dry electrophoretic transfer cell, and the membranes were blocked in Tris-buffered saline (20 mM Tris, 125 mM NaCl, 0.1% Tween 20) with 5% nonfat-dried milk at 4°C overnight. The next day, the membranes were incubated with goat anti-rat HO-1 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) in 1:2000 dilution for 2 h at room temperature. After 4-times washing, the membranes were incubated with 1:3000 concentration of peroxidase-conjugated anti-rabbit/goat immunoglobulin (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) as the secondary antibody for 1 h followed by washing steps. The membranes were then treated with the chemiluminescent substrate luminol (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and hydrogen peroxide, and the luminescent signals were detected by exposure to Kodak BioMax XAR film. To check for equal loading of proteins, the blots were stripped off and the membranes were re-probed with rabbit anti-actin antibody (1:5000) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).

5.2.12. Glutamate cysteine ligase activity

The activity of glutamate cysteine ligase was determined by detection of the fluorescent product of the reaction of 2,3-naphthalenedicarboxyaldehyde (NDA) with γ-glutamylcysteine produced as the result of glutamate cysteine ligase activity (White et al., 2003). Approximately 30 mg tissue was homogenized in 200 µl TES/SB buffer containing 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, and 2 mM serine. The

homogenates were incubated on ice for 20 min, and centrifuged at 15,000 g for 20 min at 4°C. Fifty µl of the supernatants was transferred to a second series of tubes, and 50 µl of reaction cocktail containing 400 mM Tris, 2 mM EDTA, 20 mM sodium borate, 2 mM serine, 40 mM MgCl₂, 20 mM glutamic acid, and 40 mM ATP was added. After a 5 min pre-incubation, 50 µl of 4 mM cysteine (a stock solution was diluted in TES/SB) was added and samples were incubated for 45 min. Then, 50 µl of 0.8 M sulfosalicylic acid was added, the tubes were vortexed, kept on ice for 20 min, and the precipitated proteins were pelleted at 700 g for 5 min. Twenty µl of the supernatants were loaded in a black multi-well plate and 180 µl of NDA derivatization solution containing 40 mM Tris, 90 mM NaOH, 1.1 mM NDA (in DMSO), pH 12.5, (did not vortex, otherwise would be polymerized) was added. The plate was incubated for 30 min at room temperature in the dark, and the fluorescence emission of NDA-γ-glutamylcysteine was detected at 528 nm with excitation at 472 nm in a plate reader.

For assessment of the enzyme activity in liver, the same procedure as for the heart samples was used except that because the amount of the enzyme in liver is much higher than that in heart, more diluted samples were used for liver tissues. Twenty mg of tissue was homogenized in 500 µl TES/SB buffer, and the supernatants were diluted 1:15 before treating with the reaction cocktail. Furthermore, the time course of incubation with the reaction cocktail was decreased from 45 min to 10 min.

5.2.13. NAD(P)H quinone oxidoreductase activity

The activity of NAD(P)H quinone oxidoreductase (quinone reductase) was determined by assessing the ability of the tissues to reduce 2,6-dichloroindophenol (DCIP) (Spencer & Rifkind, 1990). About 30 mg of tissue was homogenized in 200 µl of 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin, and incubated on ice for 10 min. The homogenates were then centrifuged for 10 min at 9,000 g at 4 °C, and 20 µl of the supernatants was added to cuvettes containing 178 µl of buffer containing 50 mM Tris-HCl, pH 7.5, 0.07% bovine serum albumin, and 500 µM NADPH. The cuvettes were vortexed, and placed in the spectrophotometer. The first 2-3 seconds were recorded at 600 nm and then, 2 µl of 6 mM sodium DCIP (60 µM final) was added to the cuvettes and the kinetics of DCIP reduction was recorded for 2 min. The rate of DCIP reduction was also measured in the presence of the quinone reductase inhibitor,

dicoumarol, and the activity of the enzyme was determined from the difference of enzyme activity in the presence and absence of dicoumarol. Calculations were made using an extinction coefficient of $21 \text{ mM}^{-1}\text{cm}^{-1}$ for DCIP.

For liver tissues, the assay was performed by the same protocol except that the lysate supernatants were diluted 10 times, and then 30 μl of the diluted supernatants was used in the reaction.

5.2.14. Tissue ATP content

The ATP level in heart tissues was determined by a luciferase method using a bioluminescence kit (Sigma, Saint Louis, MO, USA). Thirty mg of the tissue was lysed in RIPA buffer containing 50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF, 1mM DTT, 5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.8, for 30 min on ice, and centrifuged at 12,000 g for 10 min at 4°C. Then, 70 μl of the ATP assay mixture containing luciferase, luciferin, MgSO_4 , DTT, EDTA, bovine serum albumin, and tricine buffer salts was added to the wells of a white multi-well plate, left for 3 min and then 70 μl of the tissue lysate supernatant was added, mixed, and the luminescence light was detected in a luminometer.

5.2.15. Total polyphenol content

Total amount of polyphenols in saskatoon berries, green tea extract, and broccoli sprouts was measured based on transfer of electrons in alkaline medium from phenolic and other antioxidant compounds present in plant extracts to phosphomolybdic/phosphotungstic acid complexes in the Folin-Ciocalteu reagent, forming blue complexes which can be detected by a spectrophotometer (Ainsworth & Gillespie, 2007). To extract polyphenols, saskatoon berries and broccoli sprouts in power form and green tea extract were mixed 1/100 (w/v) with 95% methanol and left at room temperature for 48 h in the dark. They were centrifuged at 13,000 g for 5 min and the supernatants were transferred to new tubes. The tea supernatant was diluted 1/50 with 95% methanol, and 100 μl of the sample supernatants and standards (gallic acid in methanol) was taken, 200 μl of 10% Folin-Ciocalteu reagent was added, vortexed and left to stand for 1-2 min. Then, 800 μl of 700 mM Na_2CO_3 was

added, mixed, and incubated at room temperature for 2 h, and the developed blue colour was measured at 765 nm.

5.3. Statistics

Data shown are means \pm SEM. Statistical analysis was performed with SPSS software using one-way analysis of variance (ANOVA). For ischemic-reperfused groups, comparisons were made between animals in the treatment groups (berries, tea, and sprouts) and non-treated ischemic-reperfused animals (control) using 2-sided Dunnett as the post hoc test. Comparison between non-treated ischemic-reperfused animals (control) and non-ischemic animals (sham) was made by paired Student's t-test. Data on LDH release were analyzed by repeated measurements ANOVA. The statistical outliers (3 data points) were identified with an outlier calculator at <http://www.graphpad.com/quickcalcs/Grubbs1.cfm> and excluded from analysis. A *p* value less than 0.05 was considered significant. Percent increases or decreases were also calculated for the treatment groups relative to the difference between control ischemic-reperfused and sham groups.

5.4. Results

5.4.1. Cell death measurements

To determine the extent of cell death induced by ischemia-reperfusion, 3 different approaches were used: lactate dehydrogenase in heart effluents, and caspase-3 activity as well as DNA fragmentation in heart tissues. The degree of necrosis in the heart tissues was determined by measuring the amount of lactate dehydrogenase (LDH) released into the perfusate. As LDH is a cytosolic enzyme, its release into the perfusates indicates disruption of cell membrane which is a characteristic of the necrotic type of cell death. In clinical settings, elevation of plasma levels of LDH is used as a diagnostic test for myocardial infarction.

LDH release was elevated during the initial minutes of reperfusion, remained relatively low (although still higher than the pre-ischemic minutes) between minutes 5 and 35 of reperfusion, and began to rise afterwards (Figure 5.1). Since LDH did not increase

during the stabilization period, it is unlikely that the few minutes interval between the excision of hearts and cannulation on the apparatus has largely damaged the heart cells. In the sprouts group, it seems that LDH release remained relatively low for a more prolonged period of time, although it started to increase around minute 60 of the reperfusion. The increase in LDH release occurred faster in the berries and tea groups in comparison with the control. Although the tea group initially seemed to be similar to the control group, by the end of the experiment it showed a sharp increase in the released LDH. LDH release in the sprouts group, however, remained lower compared to the control. By the end of the reperfusion time the difference between treatment groups and control became more pronounced. However, none of the berries, tea, or sprouts groups significantly differed from the control at any time point. As hearts from the sham group were harvested right after the stabilization period, there is no information from them in this experiment.

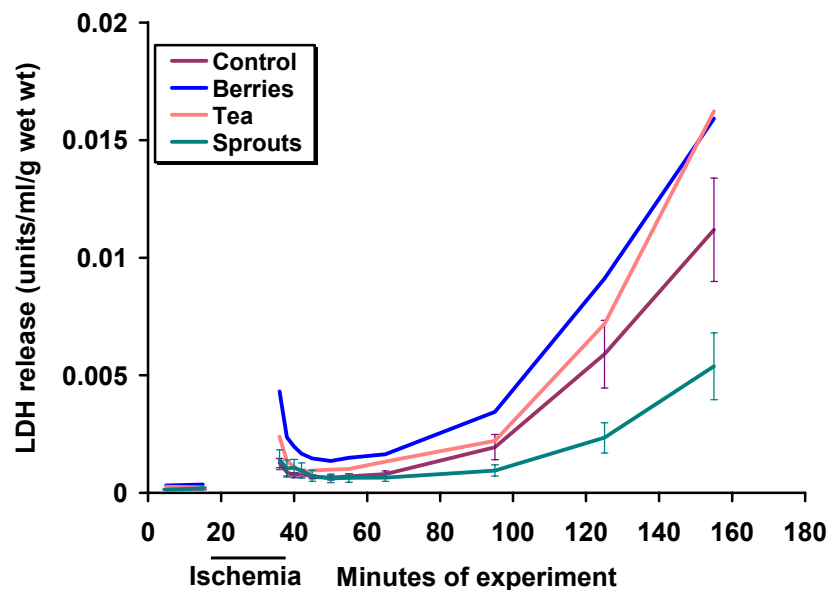


Figure 5.1. Lactate dehydrogenase (LDH) released into the heart effluents.

Aliquots of the coronary effluents were collected during 15 min stabilization period and 2 h reperfusion. The amount of LDH was determined and expressed as units of LDH present in the effluents per ml of the perfusate per gram of the wet tissue. Each point represents means \pm SEM. The experimental groups consisted of: control $n=7$; berries $n=6$; tea $n=6$; and sprouts $n=10$. Because the error bars were tall, for simplicity only those from control and sprouts groups are presented. There was no significant difference between control and any of the other groups at any time point.

Apoptosis in the heart tissues was determined using examination of caspase-3 activity, one of the important effector caspases involved in apoptosis. Ischemia-reperfusion increased the activity of caspase-3 by 270% (Figure 5.2). All of the treatment groups showed a decrease in caspase activity, ranging from 43 to 86 percent, but only the decreases in the tea (85%) and sprouts (86%) groups were significant.

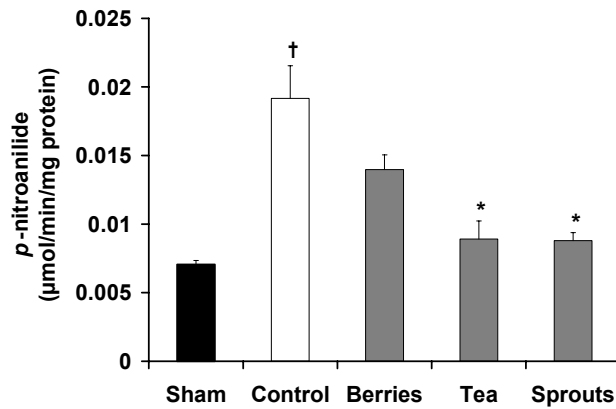


Figure 5.2. Caspase-3 activity in heart tissues.

The activity of caspase-3 in the hearts was determined by quantification of produced *p*-nitroanilide, the cleaved caspase-3 substrate. The bars represent means \pm SEM. The number of animals per experimental group was as follows: sham $n=5$; control $n=8$; berries $n=6$; tea $n=6$; sprouts $n=10$. † is for $p<0.05$ vs. sham. * shows $p<0.05$ vs. control.

DNA fragmentation in the heart tissues was quantified as another indicator of cell death. DNA fragmentation showed a 1.9-fold increase in the control compared to the sham group (Figure 5.3). Although all treatment groups had a lower (66-78%) DNA fragmentation *versus* the control, only the sprouts group was found to be significant. Both berries and tea groups exhibited DNA fragmentation as low as sprouts; however their difference was not significant, perhaps because of lower statistical power due to merely 4 animals per group in this measurement.

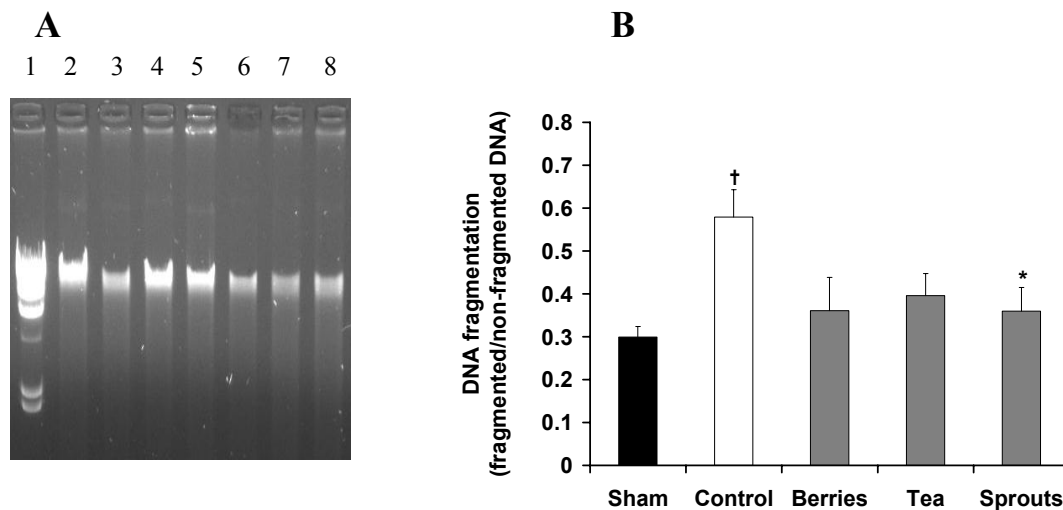


Figure 5.3. DNA fragmentation in heart tissues.

DNA was extracted from the hearts and exposed to agarose gel electrophoresis. Then, the gels were imaged and scanned for densitometric analysis of the separated DNA.

A. Gel electrophoresis of the DNA. Lanes are as follows: lane 1, *Hind*III-digested lambda phage DNA; lane 2, DNA extracted from sham group; lanes 3 and 4, DNA from sprouts group; lane 5, DNA from tea group; lane 6, DNA from berries group; lanes 7 and 8, DNA from control group.

B. Densitometric analysis of gel images. The amount of DNA has been expressed as the ratio of fragmented to non-fragmented DNA. The values represent means \pm SEM. Some of samples from each groups were lost due to technical difficulties occurred during performing the assay. The number of animals in each bar was as follows: sham n=4; control n=6; berries n=4; tea n=4; sprouts n=6. † shows $p < 0.05$ vs. sham. * indicates $p < 0.05$ vs. control.

5.4.2. Assessment of oxidative stress

To assess ischemia-reperfusion-induced oxidative stress in cardiac cells, two indicators of oxidative stress were measured: TBARS and aconitase activity. TBARS is a general test for oxidative damage. It detects malondialdehyde and other reactive aldehydes present in the samples through its reaction with thiobarbituric acid and development of a colorimetric product that can be identified by spectrophotometer. Ischemia-reperfusion caused a 60% increase in reactive aldehydes as revealed from comparison of the control with the sham group (Figure 5.4). Treatment groups showed up to 100% inhibition, with berries (73%) and sprouts (complete inhibition) being statistically significant.

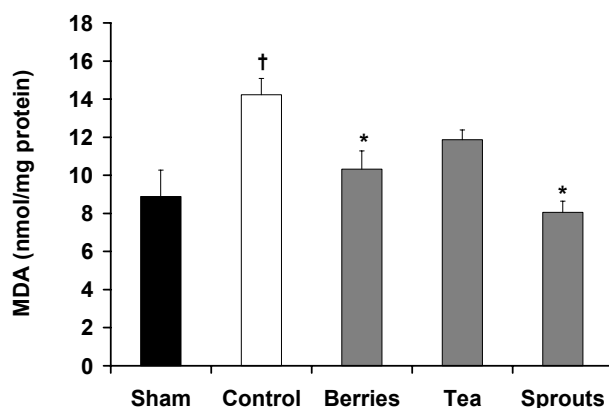


Figure 5.4. Lipid peroxidation in heart tissues.

Reactive aldehydes in the heart tissues were measured using the TBARS assay, with malondialdehyde (MDA) as the calibration standard. Columns are means \pm SEM. The number of animals in each group was as follows: sham $n=5$; control $n=8$; berries $n=6$; tea $n=6$; sprouts $n=10$. † shows $p<0.05$ vs. sham. * is for $p<0.05$ vs. control.

Aconitase is one of the enzymes in the Krebs cycle of mitochondria responsible for conversion of citrate to isocitrate with the intermediate formation of *cis*-aconitate. Aconitase has an iron-sulfur centre in its active site which is sensitive to oxidation and loss of iron (Han et al., 2005). As ROS have been shown to inactivate aconitase, aconitase activity can be considered as a marker of oxidative stress. Aconitase activity was lost by 12.5 % in the control compared to the sham group (Figure 5.5). Treatments preserved the enzyme activity by 66 to 82 percent, but only the sprouts group (82%) was significant.

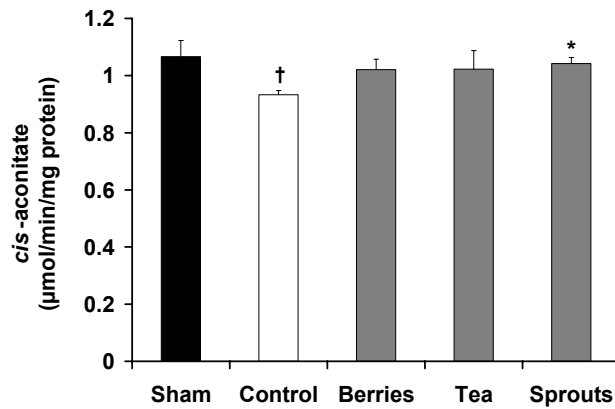


Figure 5.5. Aconitase activity in heart tissues.

Aconitase activity was determined based on the ability of the tissue samples to produce *cis*-aconitate from citrate present in the reaction mixture. The bars show means \pm SEM. The number of animals in each group was as follows: sham n=5; control n=8; berries n=6; tea n=6; sprouts n=10. † shows $p < 0.05$ vs. sham. * indicates $p < 0.05$ vs. control.

5.4.3. Tissue glutathione levels

Glutathione (GSH) is one of the most important non-enzymatic endogenous antioxidants in the cytosol, and has been introduced as one of the important antioxidants acting against reperfusion-induced oxidative stress (Haramaki et al., 1998). The level of GSH in the heart tissues decreased significantly by 37% after ischemia-reperfusion (Figure 5.6). None of the treatments gave statistically significant improvement. Glutathione disulfide (GSSG) in the hearts was not significantly affected by ischemia-reperfusion or by any of the treatments (Figure 5.6).

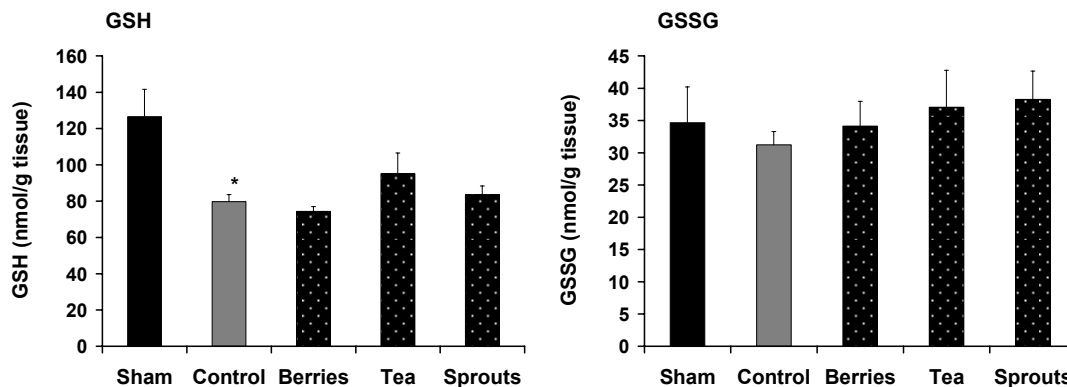


Figure 5.6. Reduced (GSH) and oxidized (GSSG) glutathione in heart tissues.

The bars show means \pm SEM. The number of animals in each group was as follows: sham $n=5$; control $n=8$; berries $n=6$; tea $n=6$; sprouts $n=10$.

GSH. The tissue level of GSH was determined with DTNB as described in Materials and Methods. * indicates $p < 0.05$ vs. sham.

GSSG. The tissue level of GSSG was determined by oxidation of NADPH in the presence of glutathione reductase as described in Materials and Methods. No significant difference was detected between GSSG of different groups.

Similarly, there was no significant difference between groups in the GSH to GSSG ratio, although ischemia-reperfusion caused a non-significant 38% decrease in the control compared to the sham group (Figure 5.7). The total amount of glutathione in the heart tissues was significantly decreased by 27% in the control group, but none of the dietary treatments was protective (Figure 5.7).

The levels of glutathione in the liver of animals who had been fed with either regular (control) diet or any of berries, tea, or broccoli sprouts diets were also examined and the sprouts group had a significant 12% increase in the total glutathione (Figure 5.8).

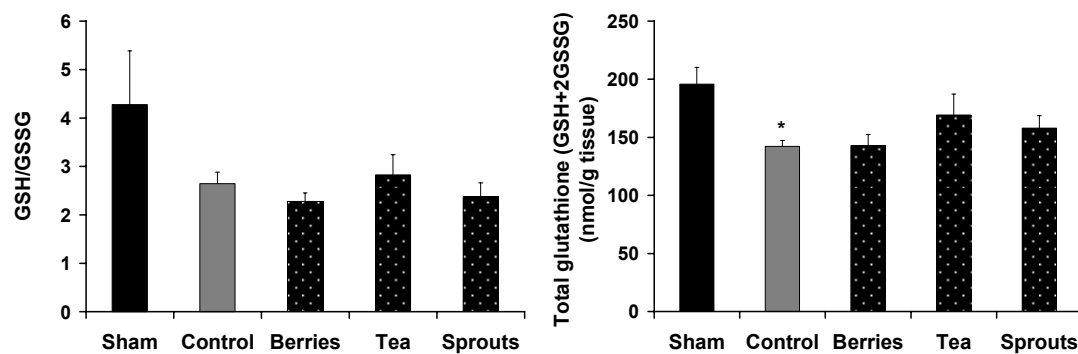


Figure 5.7. Ratio of glutathione (GSH) to glutathione disulfide (GSSG) and total amount of glutathione in heart tissues.

Data represent means \pm SEM. The number of animals in each group was as follows: sham n=5; control n=8; berries n=6; tea n=6; sprouts n=10. * shows $p < 0.05$ vs. sham.

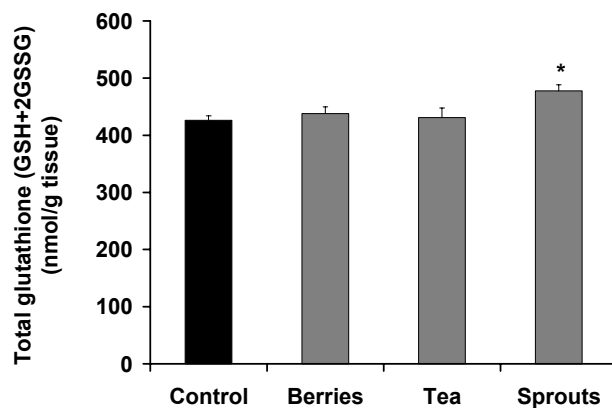


Figure 5.8. Total glutathione in liver tissues.

Bars are means \pm SEM. The number of animals in each group was as follows: control n=8; berries n=6; tea n=6; sprouts n=10. * is for $p < 0.05$ vs. control.

5.4.4. Evaluation of phase 2 enzymes

In order to see whether some of the protection of the dietary pre-treatments had been rendered through induction of phase 2 enzymes, the activities of glutamate cysteine ligase and quinone reductase as well as the protein level of heme oxygenase-1 were determined. The enzyme glutamate cysteine ligase is the rate-limiting enzyme in the synthesis of glutathione, and so its induction might enhance cellular glutathione content and consequently the antioxidant potential of cells. Ischemia-reperfusion caused a 28% decline in the activity of glutamate cysteine ligase in hearts (Figure 5.9). Sprouts and berries did not prevent the loss of the activity, but tea caused a 78% significant amelioration. In liver, while all of the treatments seemed to elevate the activity of glutamate cysteine ligase, only the sprouts showed a statistically significant 40% increase (Figure 5.9).

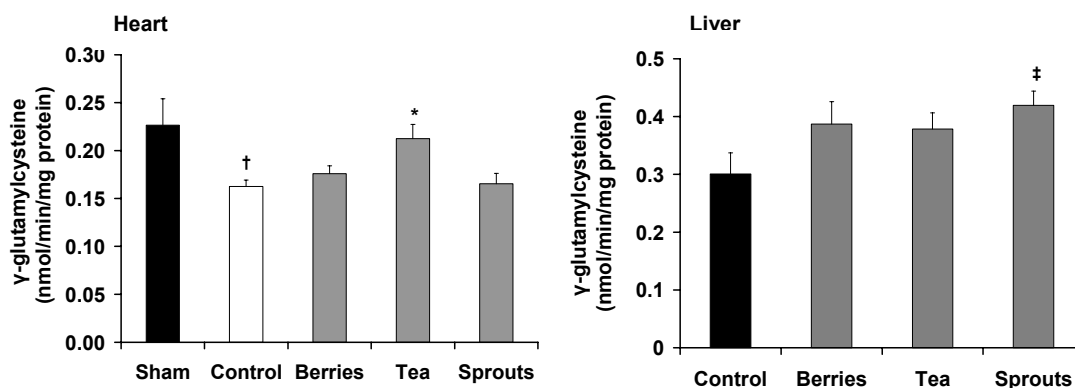


Figure 5.9. Glutamate cysteine ligase activity in heart and liver tissues.

The activity of glutamate cysteine ligase was determined by fluorometric detection of the product, γ -glutamylcysteine using the procedure described in Materials and Methods. Bars are means \pm SEM. The number of animals in each group was as follows: sham $n=5$; control $n=8$; berries $n=6$; tea $n=6$; sprouts $n=10$. † shows $p<0.05$ vs. sham. * shows $p<0.05$ vs. control. ‡ is for $p<0.05$ vs. control.

Ischemia-reperfusion caused a non-significant decrease in the activity of quinone reductase in hearts (Figure 5.10). Hearts in the tea group had a statistically significant 147% protection against this decrease. In liver, sprouts increased the activity of quinone reductase by 44% in comparison with the control (Figure 5.10). Measuring heme oxygenase-1 protein in heart, there were no statistically significant differences between any of the groups (Figure 5.11).

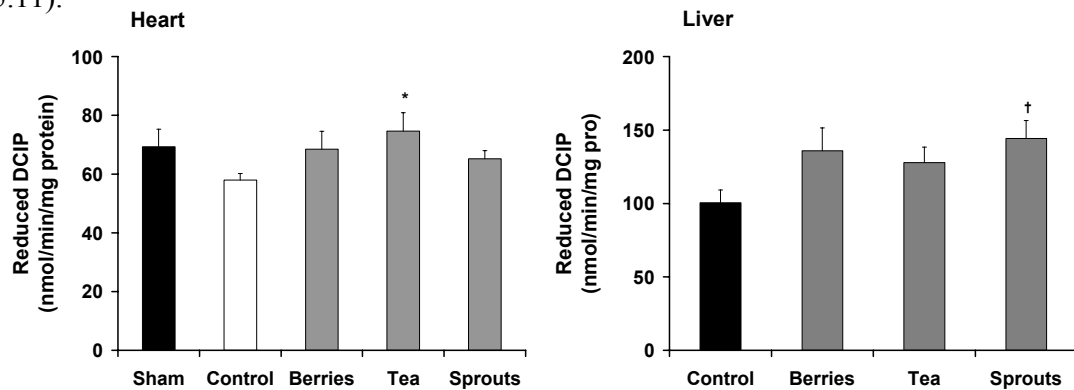


Figure 5.10. Quinone reductase activity in heart and liver tissues.

The activity of quinone reductase was determined by measuring the rate of DCIP reduction in the presence of NADPH. Bars are means \pm SEM. **Heart**, The number of animals in each group was as follows: sham n=5; control n=7; berries n=6; tea n=6; sprouts n=10. * shows $p < 0.05$ compared to control. **Liver**, The number of animals in each group was as follows: control n=7; berries n=5; tea n=6; sprouts n=10. † shows $p < 0.05$ vs. control.

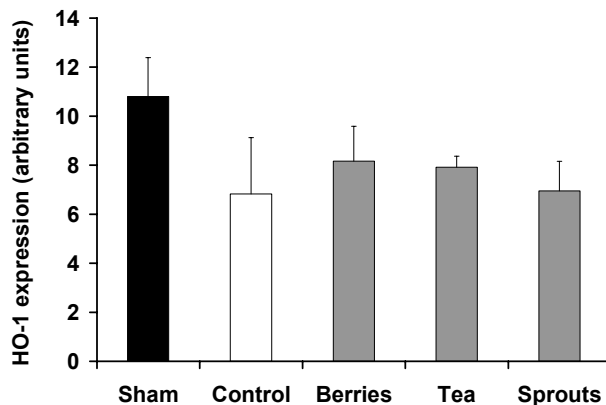


Figure 5.11. Heme oxygenase-1 protein expression in heart tissues.

Samples of the heart tissues were analyzed for heme oxygenase-1 by western blotting as described in Materials and Methods. Columns are means \pm SEM. The number of animals in each group was as follows: sham n=5; control n=8; berries n=6; tea n=6; sprouts n=10. No significant difference was observed between groups.

5.4.5. Heart ATP levels

Measuring tissue ATP revealed very low levels of ATP in the ischemic-reperfused groups compared to the sham animals (Figure 5.12). However, sprouts showed a significant preservation of tissue ATP content compared to the ischemic-reperfused control group.

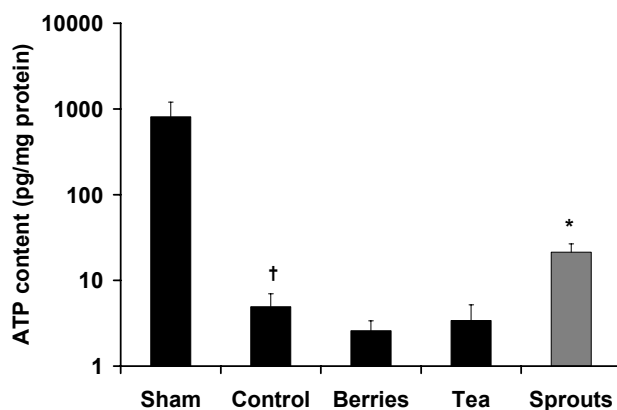


Figure 5.12. ATP content in heart tissues.

Tissue ATP levels were determined by a luminescence kit. Columns are means \pm SEM. The number of animals in each group was as follows: sham $n=5$; control $n=8$; berries $n=6$; tea $n=5$; sprouts $n=10$. † shows $p<0.05$ vs. sham. * indicates $p<0.05$ vs. control.

5.4.6. Coronary flow rate

Coronary flow rate from all of ischemic-reperfused groups had a decreasing trend during the time of the experiment (Figure 5.13). Sprouts and to a lesser extent tea showed improvements; however, at no time point were the increases statistically different from the control.

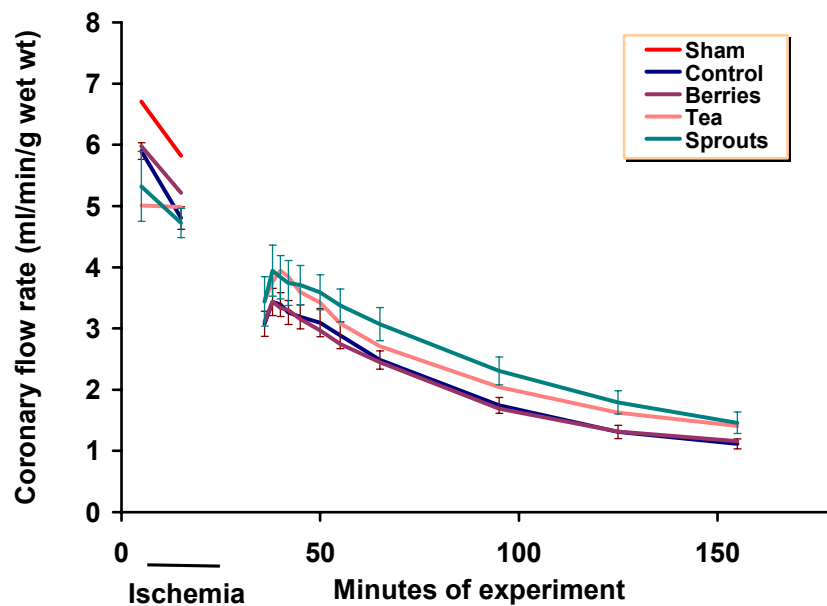


Figure 5.13. Coronary flow rate during the stabilization and reperfusion time.

Coronary flow rate was determined by measuring the amount of heart effluents per minute divided by the wet weight of heart. Each point represents means \pm SEM. The number of animals in each group was as follows: sham n=5; control n=7; berries n=6; tea n=6; sprouts n=10. For better view only standard error bars of the control and sprouts groups have been shown. There was not significant difference between groups.

5.4.7. Total polyphenol content

To compare the polyphenol content of the diets, and to see whether the effects exhibited by different dietary interventions could be related to the phenolic content of the feeds, the polyphenol content of saskatoon berries, green tea extract, and broccoli sprouts was determined. Green tea extract had almost 50 times more polyphenols than saskatoon berries and broccoli sprouts that is reasonable, as unlike saskatoon berries and broccoli sprouts, green tea extract contained concentrated polyphenols (Table 5.2). The polyphenol content of broccoli sprouts was the lowest among the three plant materials.

Table 5.2. Total polyphenol content in saskatoon berries, green tea extract, and broccoli sprouts, and the calculated amount of polyphenol added to the diets.¹

	Gallic acid equivalents (nmol/mg plant dry weight)	% of diet	Polyphenol content of diets (μ mol/kg)
Saskatoon berries	3.95 \pm 0.08	5.0%	1.97 \pm 0.04
Green tea extract	165.2 \pm 1.68**	0.25%	4.13 \pm 0.04**
Broccoli sprouts	2.90 \pm 0.14*	2.0%	0.58 \pm 0.03*

¹ Data are the mean \pm SEM.

* indicates $p < 0.05$ vs. broccoli sprouts.

** indicates $p < 0.05$ vs. saskatoon berries and broccoli sprouts.

5.5. Discussion

We hypothesized that flavonoids would reduce myocardial cell death caused by ischemia and reperfusion through multiple mechanisms including attenuation of oxidative stress by their direct antioxidant activities and indirectly by induction of antioxidant and phase 2 enzymes. The results of the study herein showed that 10 days consumption of saskatoon berries, tea, or broccoli sprouts in the diet before ischemia and reperfusion gave significant protection as evidenced by lower cell death and mitigated the extent of oxidative stress. Sprouts and tea decreased cell death by up to 86%, as assessed by caspase-3 activity and/or DNA fragmentation. Sprouts and berries ameliorated oxidative stress alterations in lipids, as evaluated by TBARS (116 and 73 percent decrease, respectively), and sprouts gave 82% protection to the protein aconitase. Tea was the only intervention that prevented the decrease in activities of the two phase 2 enzymes, glutamate cysteine ligase and quinone reductase, in ischemic-reperfused hearts. In liver, the only dietary treatment that caused a significant change in phase 2 enzyme levels was sprouts which showed a 40% increase in glutamate cysteine ligase activity and a 44% increase in quinone reductase activity.

Cell death in ischemic-reperfused hearts occurs by both apoptosis and necrosis (Zhao & Vinten-Johansen, 2002). Both forms of cell death were tested in our study; lactate dehydrogenase as a marker of necrosis and caspase-3 activity and DNA fragmentation as representatives of apoptosis. None of the treatments had significant effect on necrosis, as measured by lactate dehydrogenase release into the heart effluents. Two of the treatments, berries and tea, seemed to worsen lactate dehydrogenase release, while sprouts appeared to be protective. None of these effects was statistically significant. Nevertheless, this poses the possibility that berries and tea may promote necrosis, and opens up an area for further investigation of possible effects of the compounds, either flavonoids or non-flavonoids, in berries and tea in stimulation of the necrotic form of cell death. Our results with lactate dehydrogenase release in heart effluents is in disagreement with reports from other investigators indicating that 10 days or 1 hour pre-treatment with catechin (Modun et al., 2003) or 3 weeks (Al Makdessi et al., 2006) or 10 min (Fantinelli et al., 2005) pre-treatment with proanthocyanidins (polymers of catechins) significantly decreased lactate dehydrogenase release from hearts. The reason for this inconsistency is not clear to us, but there are some possible explanations. For example, the dose of catechin used by Modun and colleagues was 2.5 times the dosage of green tea catechins applied in our study.

Furthermore, the green tea extract used by us contained additional types of catechins such as epigallocatechin gallate which are more prone to cytotoxicity than catechin itself (Ohshima et al., 1998).

Against apoptosis, as measured by caspase-3 activity, green tea extract and broccoli sprouts protected very well. Berries did not inhibit activation of caspase-3 significantly, although their effects did approach significance ($p=0.07$). Also, berries and tea groups did not show a significant lower DNA fragmentation, but this might have been due to low number of samples in this experiment as they appeared to prevent DNA fragmentation as effectively as sprouts. Several groups of investigators have reported the effect of catechins in limiting apoptosis (Townsend et al., 2004; Hirai et al., 2007) and infarct size (apoptosis and necrosis) (Suzuki et al., 2007; Potenza et al., 2007; Townsend et al., 2004; Aneja et al., 2004). Anthocyanins from black soybean seed coat (Kim et al., 2006) and maize kernels (Toufektsian et al., 2008) also showed the potential to reduce infarct size. Recently, broccoli, but not broccoli sprouts, has demonstrated to attenuate myocardial cell apoptosis after ischemia-reperfusion (Mukherjee et al., 2008).

All dietary treatments markedly prevented at least one of the biochemical changes caused by ischemia-reperfusion-induced oxidative stress. Malondialdehyde as an indicator of lipid peroxidation was diminished especially in berries and sprouts groups. The activity of oxidative stress-sensitive aconitase was also preserved by sprouts. Similar to our results, Amorini et al. (2003) found that reperfusion of isolated hearts with cyanidin-3-glucopyranoside in the perfusate decreased production of malondialdehyde. Other investigators have reported the ability of catechin (Modun et al., 2003) and proanthocyanidins (Fantinelli et al., 2005; Sato et al., 1999) to prevent lipid peroxidation in heart ischemia-reperfusion. Likewise, 3 weeks of feeding rats with oligomeric proanthocyanidins from grape seeds decreased hydroxyl radicals (Pataki et al., 2002) and malondialdehyde (Sato et al., 1999) released into post-ischemic heart effluents.

Despite the fact that the treatments substantially mitigated oxidative stress-induced changes in lipids and proteins, they did not preserve cellular reduced glutathione, nor improve the GSH to GSSG ratio, a biomarker which closely reflects cellular redox state (Wu et al., 2004a). The reason for the lack of the effect of treatments on glutathione is not clear, but the task of glutathione in detoxification reactions may be implicated. Dietary treatments in our study contained flavonoids. Oxidized flavonoids have electrophilic properties, and can be detoxified through conjugation with glutathione. Therefore, flavonoids might have

sequestered some of the cellular glutathione after being oxidized in oxidative conditions of ischemia-reperfusion. Formation of flavonoid-glutathione adducts has been shown in the presence of hydrogen peroxide and peroxidase (Galati et al., 2001; Moridani et al., 2001; Awad et al., 2000).

The sprouts group had elevated total glutathione in liver which was also in agreement with increased liver activity of the enzyme synthesizing glutathione, glutamate cysteine ligase, in this group. This means that it is plausible that treatment with broccoli sprouts enhance glutathione synthesis in liver, the main location of glutathione synthesis in the body, and glutathione could then be exported from the liver to other organs in need, e.g. the heart when being challenged with ischemia-reperfusion-induced oxidative stress. Unfortunately, the *ex vivo* condition of our study did not allow us to examine this.

Whereas the sprouts group had higher glutamate cysteine ligase activity in liver, the tea group showed a higher activity of this enzyme in heart. However, the elevation of glutamate cysteine ligase activity in hearts from the tea group could not be confirmed by the enhancement of glutathione markers in hearts, although they had relatively (non-significant) higher mean values for GSH, GSH/GSSG, and total glutathione compared to the other treatments. The higher activity of glutamate cysteine ligase in hearts of the tea group could be due to induction of the corresponding gene or inhibition of the loss of the enzyme activity following ischemia-reperfusion.

The activity of quinone reductase followed the same pattern as glutamate cysteine ligase, as sprouts and tea groups exhibited higher activities in liver and heart, respectively. Interestingly, in liver, activities of both phase 2 enzymes were comparably higher in the treatment groups than in the control group, although it was only the sprouts group which showed a statistically significant elevation, perhaps due to the higher number of animals in this group. Although saskatoon berries failed to elevate activities of the two examined phase 2 enzymes in our study, an increased induction of the glutamate cysteine ligase gene by a mixture of berries in the brain and muscle, but not the liver, of mice has been reported before (Carlsen et al., 2003). The mechanism of induction of phase 2 enzyme activities is yet unclear, but it has been suggested that inducers of the phase 2 genes likely react with a sensor molecule (possibly the Nrf2-Keap 1 complex) in the cell, modifying sulfhydryl groups through oxidation or alkylation (Dinkova-Kostova et al., 2001). Keap-1, the molecule that sequesters Nrf-2 in the cytosol, contains 9 highly reactive sulfhydryl residues

which are postulated to be critical for recognition by and reaction with phase 2 enzyme inducers.

The expression of heme oxygenase-1 in heart tissues did not show a significant change between groups, which may be at least partly due to high within-group variability in this assay. There is no information to date on the ability of catechins or anthocyanins to induce or prevent loss of heme oxygenase-1. However, there are some reports of resveratrol induction of heme-oxygenase-1 in ischemic-reperfused hearts (Kaga et al., 2005; Das et al., 2006). Broccoli has also shown to inhibit ischemia-reperfusion-induced reduction of heme oxygenase-1 (Mukherjee et al., 2008).

The ischemic-reperfused control group for many biomarkers such as glutathione parameters and phase 2 enzymes displayed lower values than the sham animals. Although these lower quantities could have been due to the actual impact of ischemia-reperfusion on the cells, it is also possible that the diminutions were because some cells in the ischemic-reperfused control group had compromised cell membrane and therefore cytoplasmic components of such cells have leaked to the extracellular environment (i.e. heart effluents) and been removed (Ferrari et al., 1990). Although the biomarkers were always expressed per protein content or weight of tissue, this possibility still exists as the damaged cells may have not been depleted of structural proteins as they have of enzymatic proteins and non-enzymatic intracellular components (e.g. glutathione). However, this possibility is not corroborated by the results from oxidative stress markers, such as caspase-3 activity, DNA fragmentation, and TBARS, which increased in the ischemic-reperfused control group instead of decreasing due to the loss from the cells. Nevertheless, it is possible that these markers have partially been lost in the heart effluents but their elevation in the heart following ischemia-reperfusion has covered this loss.

None of the treatments significantly prevented the gradual declination in the flow rate during the experiment. Sham hearts were not kept on the apparatus until the end of the experiment, so no information is available on their perfusion rate. However, as the decrease in the flow rate of the sham and other animals began in the pre-ischemia minutes, i.e. after cannulation of hearts on the apparatus, it is likely that if there was a sham group which was perfused for the whole time of the experiment, the coronary flow rate would yet have such a decline. Such a decline in the flow rate of the sham hearts was observed in the pilot work of this study where hearts from sham animals were perfused for longer periods of time, e.g. 1 or 2 h. The gradual depression in the flow rate could be due to partial obstruction of the

coronary vasculature as a result of artificial perfusion and lack of normal heart contractility and physiological vessel tonicity. Improved coronary flow in ischemic-reperfused hearts upon feeding animals with epigallocatechin gallate (Potenza et al., 2007) and proanthocyanidins (Pataki et al., 2002) has previously been reported.

Except for the sprouts, the other treatments failed to improve cellular ATP level. The improved ATP content by broccoli sprouts is in concordance with the attenuated (non-significant) amount of necrosis in the sprouts group which was evidenced by the lower level of lactate dehydrogenase released into the heart effluents. Similarly, ineffectiveness of berries and tea to prevent depletion of heart tissue ATP content was consistent with the higher (non-significant) occurrence of necrosis in these groups compared to the sprouts group. In contrast to our results with berries and green tea extract, preservation of ATP in ischemic-reperfused hearts has previously been reported for cyanidin-3-glucopyranoside (Amorini et al., 2003), epigallocatechin gallate, and gallocatechin gallate (Hirai et al., 2007).

The drastic difference in ATP content between sham and ischemic-reperfused groups was not only because the sham animals did not have an ischemic period, but it was also because the sham hearts were not kept on the apparatus as long as the ischemic-reperfused hearts. It is likely that if hearts from the sham group had been perfused for the whole time of the experiment, their cellular ATP content would gradually decline due to a gradual depression in the flow rate as a result of coronary vasculature dysfunction that impairs the access of cardiac cells to nutrients and oxygen.

Our study was the first to report protection by broccoli sprouts on hearts under conditions of ischemia-reperfusion, although the cardioprotective ability of broccoli in ischemic-reperfused hearts has recently been shown to be correlated with induction of phase 2 enzymes and activation of survival pathways (Mukherjee et al., 2008). Beneficial effects of broccoli sprouts on other types of oxidative stress than ischemia-reperfusion have previously been reported. For instance, dietary broccoli sprouts decreased aging-related degenerative changes in hypertensive rats (Noyan-Ashraf et al., 2005) and mitigated hypertension-induced oxidative-related changes in cardiovascular and kidney tissues (Wu et al., 2004b). Also, topical application of broccoli sprouts extract to mice induced phase 2 enzymes in skin and alleviated ultraviolet radiation-induced inflammation (Talalay et al., 2007).

As the polyphenol content of broccoli sprouts was lower than saskatoon berries and green tea extract, it is unlikely that the advantages observed herein from broccoli sprouts

were only due to the polyphenols. However, at least some of the benefits observed in the tea group, including enhancement of phase 2 enzyme activities in hearts, could be because of high polyphenolic content in this concentrated extract.

Generally speaking, the beneficial effects of broccoli sprouts are attributed to glucosinolates, the precursors of isothiocyanates, some of which such as sulforaphane are potent phase 2 enzyme inducers (Fahey et al., 1997). Broccoli seeds possess high glucosinolate content (Pereira et al., 2002). As the seeds germinate, the amount of glucosinolate decreases with further reduction with the age of the sprouts. The amount of glucoraphanin, the glucosinolate form of sulforaphane, is higher sometimes by 10-100 times in young sprouts than in the mature plant (Fahey et al., 1997). Broccoli also possesses high levels of antioxidants, including vitamin C and flavonoids such as quercetin (Moreno et al., 2007; Miesan & Mohamed, 2001). The quantity of antioxidants is higher in sprouts than in the mature plant (Moriyama & Oba, 2004).

No significant toxicity has been reported in phase 1 studies with broccoli sprout extracts containing either glucosinolates or isothiocyanates (Shapiro et al., 2006). One week consumption of 100 g/day fresh broccoli sprouts decreased oxidative stress markers in plasma and urine of healthy subjects (Murashima et al., 2004). These findings and the current study suggest that consuming broccoli sprouts in daily diet may be a safe strategy to prevent unforeseen cardiac events in at risk individuals. The doses in humans of broccoli sprouts (80-100 g fresh/day) and berries (150 g fresh/day) corresponding to those used in our study are achievable by a regular diet, and given that the both dosages were relatively low the possibility of adverse side effects seem unlikely.

5.6. Conclusions

1. Broccoli sprouts gave the best protection, protecting against apoptosis and oxidative stress, and helping preserve ATP levels.
2. The protection by broccoli sprouts was likely not due to polyphenols, since they had the lowest levels among the treatments.
3. Green tea extract, which had the highest polyphenol content, could confer some protection against apoptosis and loss of phase 2 enzymes in ischemic-reperfused tissues.

4. Saskatoon berries could give some protection against oxidative stress (TBARS formation) and indications of protection against apoptosis, but otherwise were less protective than the sprouts or green tea extract under these conditions and doses tested.

5. Whether any of the treatments acted by inducing phase 2 enzymes was inconclusive, but green tea extract could help preserve phase 2 enzymes in heart while broccoli sprouts could increase their levels in liver.

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CHAPTER 6

CONCLUDING REMARKS

6.1. General discussion

As flavonoids possess a variety of biologic properties, it is plausible that they operate through multiple mechanisms. Acting as antioxidants, flavonoids can directly scavenge reactive oxygen species, but meanwhile due to the redox changes that they cause in the cellular environment they may also alter signaling pathways to induce endogenous cellular antioxidants. The data presented herein showed that flavonoids differ not only in the strength but also in the mechanism of the antioxidant activity. For instance, in *in vitro* experiments catechins were more effective than anthocyanins in prevention of damage caused by both ischemia-reperfusion and *t*-buOOH. However, in ischemia-reperfusion different catechins decreased cell death to similar degrees, while in *t*-buOOH-treated cells epigallocatechin gallate was nearly twice as protective as catechin, and proanthocyanidins were ineffective. Similarly, whereas quercetin exhibited very mild protection in ischemic-reperfused cells, it caused the most powerful protection among the polyphenols tested in *t*-buOOH-treated cells. Ascorbate showed similarly different actions in ischemia-reperfusion and *t*-buOOH circumstances; whereas it showed strong protection in the former, it was ineffective or damaging in the latter.

The reason for the different behaviour of flavonoids and ascorbic acid in the two oxidative stress models tested could be the severity of oxidative stress and more likely the type of stress. *t*-buOOH has probably caused more intensive oxidative stress than ischemia-reperfusion, validated by almost zero cell survival in many of the *t*-buOOH experiments compared to one-third to one-half cell viability in most of the ischemia-reperfusion conditions. On the other hand, in ischemia-reperfusion experiments, some of the cell death occurred due to lack of glucose and serum. Although these two shortages, as we discussed earlier, increase the intensity of oxidative stress, the signaling pathways that they engage to elevate reactive oxygen species are likely different from those initiated by hypoxia and from those evoked by *t*-buOOH. It is tempting to suggest that flavonoids have different abilities to

interact with various signaling pathways stimulated during any of these and other pathological stimuli. If this is true, then the best protective flavonoids against ischemia-reperfusion injury might not be those which protect against other types of oxidative stress. Moreover, given that flavonoids act in a cell- and tissue-specific manner, and considering that the goal of this and other research in the medicinal area is to improve human health, any strategies claiming a benefit must be examined in humans or at least human cells and/or tissues.

Unfortunately, due to inconsistency in the rate of cell death in H9c2 ischemia-reperfusion experiments we could not continue the project to investigate whether some of the protection exhibited by catechins and ascorbic acid resulted from induction of phase 2 enzymes. However, for catechin long- and short-term pre-treatments were tested and showed that the protective effect of catechin was increased in the long-term incubation time, posing the possibility that cellular antioxidant enzymes may have been induced and reinforced the cells' ability to contend with ischemia-reperfusion.

Consistent with the *in vitro* results, pre-treatment with catechins (i.e. green tea extract) decreased the rate of cell death in ischemic-reperfused isolated rat hearts, although catechins did not significantly prevent oxidative stress-induced changes in cellular biomolecules. A part of the catechin effects could be due to induction of phase 2 enzymes, which is in agreement with our hypothesis. Unlike green tea extract, the protective effect exhibited by berries (i.e. lower TBARS) was not mediated by induction of phase 2 and antioxidant enzymes. It is not clear what might have underlain the benefits of berries in rat hearts. Anthocyanins failed to inhibit cell death in exposure of H9c2 cells to ischemia-reperfusion or *t*-buOOH. However, this does not contradict that they have exerted protection in hearts. It is also possible that the benefits have been produced by other plant components present in the berries such as catechins (Sellappan et al., 2002) or ellagic acid (Carlsen et al., 2003). As berries showed lower impact on ischemic-reperfused hearts than tea and broccoli sprouts, one may suppose that the active components may not have been absorbed at the gastrointestinal tract. This possibility is excluded by reports demonstrating the bioavailability of anthocyanins in both aglycon and glycoside forms with rates of absorption corresponding to the dosage consumed (McGhie et al., 2003).

What caused the benefits of broccoli sprouts was left unanswered by our study. Little data are available on the chemical composition of broccoli sprouts, except for a high content of glucosinolates. It is not clear whether the protective effect exhibited by broccoli sprouts

was due to their glucosinolate content, specific polyphenols (Maeda et al., 2008), or other phytochemicals.

According to the results of the *in vitro* experiments, toxicity of flavonoids is more likely to occur in severe circumstances of oxidative stress (in our case, in *t*-buOOH conditions compared to the ischemia-reperfusion), where the reduction-oxidation reactions take place more quickly. For instance, whereas the toxicity of catechins did not appear in the ischemia-reperfusion experiments, even low concentration (25 μ M) of epigallocatechin gallate and proanthocyanidins during 3 days pre-treatment showed toxicity in the presence of *t*-buOOH (Figure 4.5). Similarly, ascorbate showed protection in the ischemia-reperfusion experiments, while it revealed toxicity in *t*-buOOH conditions. It is worthwhile to note that reductants such as ascorbate are known to decompose lipid hydroperoxides, resulting in the formation of more reactive species (Lee et al., 2001; Baysal et al., 1989). H9c2 cells as a cell line were quite resistant to the stress caused by the ischemia-reperfusion in our study, requiring 5 h or more of ischemia to produce significant cell death. It is possible that the ischemia-reperfusion has not produced a severe oxidative stress in H9c2 cells. However, in *in vivo* situations, the cells typically seem more vulnerable and therefore oxidative stress produces more harm in the cells. That is why even short (e.g. 20 min) episodes of ischemia, such as that used in our study with rats, are deadly to cardiac cells. This implies that the possible toxicity of high doses or purified flavonoids should be taken into consideration while dealing with cells *in vivo*.

6.2. Limitations and future studies

6.2.1. Cell culture

H9c2 cells were not a good type of cell to examine ischemia-reperfusion, as they showed resistance to the stress. If a comparison between different polyphenols is needed to be made *in vitro*, primary cells or a cell type more identical to the human heart is preferable.

6.2.2. *Ex vivo* model

The *ex vivo* model of heart ischemia-reperfusion has some drawbacks. It is not quite comparable to the *in vivo* conditions of ischemia-reperfusion. In response to a stress, it is not

only the harmed organ which challenges the disturbance, but the whole body responds to the damage. Antioxidants such as glutathione may be released from other organs especially liver to help the heart to survive oxidative stress. This is the fundamental principle for the remote ischemia preconditioning, a procedure in which brief ischemia in a remote area could precondition an organ against a sustained ischemia (Kantor et al., 2007). Further, excising the heart and mounting it on the Langendorff apparatus damages the heart. Hearts on the Langendorff apparatus do not beat for the entire time of the experiment and heart muscles contract less strongly. Moreover, we did not perfuse the hearts with blood, which contains platelets and leukocytes, two major contributors to the no-reflow phenomenon. Neutrophils are also an important source of reactive oxygen species. Whether our treatments show the same results in normal hearts *in vivo* needs to be examined.

6.2.3. Uncertain essence of broccoli sprouts

Feeding broccoli sprouts to rats were the most protective intervention in our *ex vivo* study. However, it is unclear what component in the broccoli sprouts was responsible for the benefits. Since the content of glucoraphanin (Fahey et al., 1997) and sulforaphane (Nakagawa et al., 2006) is at least 10 times higher in the young rather than mature broccoli sprouts, the observed benefits may have resulted from these compounds. To test this, an equal amount of sulforaphane could be administered to rats to clarify this obscurity. It is noteworthy that the same pattern for the gradual decrease of glucoraphanin during development of the plant is not probably the case for the polyphenols, as midmaturity spinach has shown higher phenolic compounds than immature and mature spinach (Pandjaitan et al., 2005).

6.2.4. High amount of catechins in green tea extract

The green tea extract showed some benefits. However, effective concentrations are not achievable by daily drinking tea. To achieve equal amounts of catechins, one should drink almost 8.5 cups of green tea or 36 cups of black tea (Khokhar & Magnusdottir, 2002). However, lower concentrations of green tea extract may still be helpful. Future studies may be directed towards examining catechins in dietary achievable concentrations or testing teas rather than the purified extracts.

6.2.5. Induction of phase 2 enzymes

Two of the dietary interventions, broccoli sprouts and green tea extract, increased or prevented loss of the activities of glutamate cysteine ligase and quinone reductase. Several directions can be taken to develop this finding. First, more phase 2 and antioxidant enzymes can be tested. Second, green tea extract preserved the activities of the two enzymes in hearts. It should be determined whether this preservation is mediated by protecting enzyme activity or increasing enzyme expression, and whether lower concentrations of the catechins can also exhibit the same effects. Third, broccoli sprouts increased activities of the enzymes in liver. It needs to be determined if this increase can be helpful for ischemic-reperfused hearts *in vivo*. Fourth, the result of the heme oxygenase-1 experiment is questionable as the standard errors were quite high. Due to technical difficulty, we could not re-examine the experiment, but more attempts are needed to confirm or reject the present results.

6.2.6. Toxicity

The toxicity of broccoli sprouts, green tea extract, and saskatoon berries was not examined in the current study. The harmlessness of these should be verified before making any recommendation to the public.

6.3. References

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